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THE ANTITUBERCULAR ACTIVITY OF ASPERGILLIC ACID AND ITS PROBABLE MODE OF ACTION

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IT HAS been reported in the recent literature[1-3] that various Aspergillus species produce inhibitory substances against *mycobacterium tuberculosis.* Because of the obvious significance of these observations, a study was undertaken in order to determine the possible inhibitory effect of the various antibiotics produced by the mold *aspergillus flavus* against the tubercle bacillus.

It has been shown during the last five years that molds belonging to the species *A. flavus* produce several antibiotic substances. White and Hill[4] showed that a moderately toxic antibiotic was produced by this mold which he named aspergillic acid. Bush and Goth[5] described a penicillin-like substance produced by an *A. flavus* which was named flavicin. Aspergillic acid inhibited a great variety of bacteria, including both gram-positive and gram-negative species. Its activity against *Myco. tuberculosis* has not been described.

On testing extracts obtained from *A. flavus* cultures against various strains of *Myco. tuberculosis* it was found that while flavicin had no inhibitory effect on this organism, aspergillic acid showed marked inhibitory properties. In addition to this finding, the experiments in which this inhibitory effect was studied threw some light on the probable mechanism of action of aspergillic acid.

MATERIAL AND METHODS

Aspergillic acid was obtained from various sources. Partially purified material was prepared in the following manner: a strain of *A. flavus* was grown on a medium containing 2 per cent bacto-tryptone,* 2 per cent dextrose† (U. S. P.), and 0.5 per cent Nacl (C. P.). Eight- to ten-day-old cultures were extracted with butyl acetate at pH 4 in a spray extraction column.[6] The active material was recovered from the solvent by shaking it with 0.1 M $NaHCO_3$ solution. The material was further purified by adsorbing the toxic impurities on charcoal (Darco G 60). Results obtained with this partially purified material were confirmed by using purified aspergillic acid.†

Two strains of *Myco. tuberculosis* were used in this study. One was the H 37 strain obtained from the American Type Culture Collection. The other was a fast-growing human strain.‡

The media for growing the tubercle bacillus were Long's synthetic medium, with and without ferric ammonium citrate (5 mg. per 100 c.c.), and bacto nutrient broth.* On the latter medium only the fast-growing tubercle

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*Difco Laboratories, Inc., Detroit, Mich.

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‡Obtained from Dr. R. C. Avery of Vanderbilt University.

bacillus multiplied readily. All media were distributed in 5 c.c. portions. Liquid media were used for the fast-growing strain: agar was added for growing the H 37 strain. Aspergillic acid was added to the media as the sodium salt.

RESULTS

When purified aspergillic acid was tested against a fast-growing strain of *Myco. tuberculosis* in broth, complete inhibition was observed up to a 1:80,000 dilution. When the same experiment was carried out in Long's synthetic medium (containing 5 mg. per 100 c.c. ferric ammonium citrate) no inhibition was observed even at 1:20,000.

Since it seemed likely that some substance in Long's medium interfered with the antitubercular activity of aspergillic acid, each one of the constituents of this medium was examined systematically for its ability to interfere with the activity of the antibiotic. When ferric ammonium citrate was excluded from the medium, the inhibitory effect of aspergillic acid was the same as in broth. Since ferric chloride had the same effect as ferric ammonium citrate, it was concluded that the ferric ion is responsible for the inactivation of aspergillic acid.

The results of a typical experiment are represented in Table I. Aspergillic acid was markedly inhibitory against the tubercle bacillus in media to which no iron was added, whereas this antibacterial effect disappeared following the addition of ferric ammonium citrate (5 mg. per 100 c.c.) to the medium. The results were evident in three days and remained unchanged for two weeks.

TABLE I.—THE INHIBITORY ACTIVITY OF PURIFIED ASPERGILLIC ACID ON A FAST-GROWING HUMAN STRAIN OF MYCO. TUBERCULOSIS

CONCENTRATION OF ASPERGILLIC ACID	AMOUNT OF GROWTH		
	NUTRIENT BROTH	LONG'S MEDIUM WITHOUT IRON	LONG'S MEDIUM WITH IRON
1:20,000	0	0	+++
1:40,000	0	0	+++
1:80,000	0	0	+++
0	+++	++	+++

While carrying out these experiments the observation was made that when aspergillic acid was added to the medium containing ferric ammonium citrate or ferric chloride, a fine red precipitate appeared within two to three minutes in the medium. This red substance, which was evidently not inhibitory, was actually taken up by the tubercle bacillus as evidenced by the bright red appearance of the colonies.

When these experiments were repeated using the slow-growing H 37 strain of *Myco. tuberculosis*, the findings were essentially the same. Aspergillic acid inhibited the growth of this organism in a medium to which no iron was added. In the presence of ferric ammonium citrate, the colonies of the H 37 strain showed an accumulation of the red substance which originated from the interaction of aspergillic acid and ferric ammonium citrate. However, these red colonies, as well as the controls to which no aspergillic acid had been added, failed to grow.

It was of interest to determine whether or not ferric ion would interfere with the antibiotic activity of aspergillic acid against organisms other than the tubercle bacillus. On testing this possibility the results were the same as

with the fast-growing strain of *Myco. tuberculosis.* Purified aspergillic acid inhibited the growth of a *Staphylococcus aureus* when added to broth in the amount of 1:80,000. Following the addition of ferric ammonium citrate (5 mg. per 100 c.c.) the inhibitory activity was not present at 1:20,000.

The growth of both strains of *Myco. tuberculosis* was much heavier in media to which iron was added than in media which had only traces of iron. This observation is in agreement with the findings of Schmidt,[7] according to whom the addition of iron to culture media stimulates the growth of *Myco. tuberculosis.*

On further study of the nature of the interaction between ferric ions and aspergillic acid, the following experiment was carried out. To 2 mg. of ferric ammonium citrate 5 c.c. of a partially purified aspergillic acid solution was added, representing 750 staphylococcus units, which correspond to approximately 10 mg. of purified material. A red precipitate appeared which was insoluble on acidification with H Cl and was readily soluble when the solution was made alkaline by the addition of 0.1 M NaOH. When the precipitate was filtered from the acidified solution, the filtrate failed to give the Prussian blue test for the presence of ferric ions. When the precipitate was ashed and subsequently dissolved in water, it gave a positive test with potassium ferrocyanide. From these qualitative tests it appeared clear that ferric ions were precipitated by aspergillic acid. Since the antibiotic activity changed simultaneously with the appearance of the precipitate, it is reasonable to assume that the red substance which appears whenever ferric ions are added to aspergillic acid represents an aspergillic acid-iron complex.

Since iron was found to interfere with the antibiotic activity of aspergillic acid, it was of importance to find out whether or not blood which contains mostly ferrous iron bound in a complex organic linkage, would interfere with the activity. To test this possibility, aspergillic acid was added in the amount of 1 mg. per cubic centimeter to defibrinated rabbit blood, which subsequently was tested for antibacterial activity. It was found that aspergillic acid added to blood still inhibited the growth of *Staph. aureus* and the fast-growing strain of *Myco. tuberculosis* up to 1:40,000, the highest dilution tested.

DISCUSSION

From the experiments reported, the following facts appear:

1. Ferric ion stimulates the growth of *Myco. tuberculosis.*
2. Aspergillic acid inhibits the growth of *Myco. tuberculosis* in media to which no ferric ions are added.
3. Ferric ions interfere with the antibiotic activity of aspergillic acid.
4. Aspergillic acid precipitates ferric ions.

The most likely explanation of these findings can be given in the following hypothesis: iron is essential for the growth of *Myco. tuberculosis;* aspergillic acid inhibits the growth by interfering with the utilization of iron or perhaps also by interfering with the functioning of iron-containing enzyme systems.

The inhibitory activity of aspergillic acid against the tubercle bacillus is sufficiently high to encourage chemotherapeutic trial in experimental animals. Such studies are being carried out at the present time in guinea pigs, and the outcome of these studies will be reported later. If these experiments indicate any in vivo antitubercular activity, clinical trials will be carried out; also, the

effect of various metals on the activity and toxicity of aspergillic acid is being investigated.

CONCLUSION

Aspergillic acid in low concentrations inhibits the growth of human strains of *Myco. tuberculosis*. Suggestive evidence is presented which indicates that the mode of action of aspergillic acid can be explained on the basis of interference with the utilization of iron by the tubercle bacillus.

The author wishes to acknowledge the technical assistance of Jane C. Neff.

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SEROLOGIC REACTIONS IN PRIMARY ATYPICAL PNEUMONIA

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PRIMARY atypical or viroid[1] pneumonia was the form of pneumonitis encountered most frequently in World War II. The clinical picture has become familiar as a result of a great many reports, and extensive studies have been made during the past few years into its epidemiologic and laboratory aspects.[2] Although the viral nature of the etiologic agent is strongly suggested by the transmission of the disease to human volunteers with bacteria-free filtrates,[3] the properties of the agent or agents are yet to be described. It is clear that the majority of instances of the syndrome are not caused by the viruses described by Weir and Horsfall[4, 5] and by Eaton and associates[6] as producing pneumonitis in the mongoose and cotton rat, respectively. Neither are they due to the influenza nor any of the other familiar viruses. However, a number of characteristic changes have been observed to develop in the sera of patients during convalescence. These have been reflected in the cold agglutinin test,[7, 8] indifferent streptococcus agglutination test,[9] elementary body type virus complement fixation test,[10-12] and in the serologic tests for syphilis.[13-15] Changes have also been reported to occur in the Weil-Felix test.[16]

The present communication is a report of the results of subjecting two or more serial specimens of serum from 100 patients, sixty-eight of whom were

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clinically diagnosed as having primary atypical pneumonia, to one or more of a "battery" of these tests. Studies were made of the cold agglutinin titer (CA), indifferent streptococcus agglutination (SA), elementary body type virus complement fixation (EB), and Kahn flocculation and Kolmer complement fixation tests for syphilis. In addition, a report is made of the results obtained when a group of seventy-three consecutively received single specimens of serum submitted for cold agglutinin testing were also examined in the sheep cell heterophil and Weil-Felix tests.

MATERIALS AND METHODS

Sera.—Sera were obtained from patients at army hospitals scattered throughout the southern half of California, Utah, Nevada, and Arizona. They were all originally submitted for cold agglutinin or influenza agglutination-inhibition antibody examinations. For the purposes of the present study, only those serial specimens of serum from patients with illnesses which had been diagnosed as "primary atypical pneumonia," "virus pneumonia," or "virus bronchitis" were included in the "primary atypical pneumonia" group. All others, which included those from patients with diagnoses of "bronchopneumonia," "pneumonitis," "lobar pneumonia," "questionable influenza," and "nasopharyngitis," were put in the "control" group. This rigid division of cases seemed necessary since in almost every instance contact between this laboratory and the actual patient was limited to correspondence with the patient's medical officer.

The hospital laboratories were requested to separate the serum from freshly clotted blood. If the blood had been stored in the cold, they were asked to bring it to body temperature before making this separation. In most instances the serum was sent to this laboratory through the regular mail with sulfanilamide as a preservative.[17] When this had not been done, the serum on arrival was transferred to such a "sulfa vial" and stored in the refrigerator until tested. The cold agglutinin (CA), elementary body virus complement fixation (EB), and serologic tests for syphilis were usually done shortly after the specimens were received. The indifferent streptococcus (SA) agglutination test on about one-half of the specimens was done from two to twelve months after receipt. This seemed justifiable since it has been demonstrated that the SA is well preserved for long periods at refrigerator temperature.[9]

Cold Agglutinin Test (CA).—The technique described by Horstmann and Tatlock[7] was followed. However, the sera were inactivated at 56° C. for fifteen minutes prior to dilution to destroy any complement which might be present. The tests were read after storage overnight in the refrigerator at approximately 0 to 4° C. and again after warming in the water bath at 37° C. for fifteen minutes. A known positive serum was included in every run as a check on the sensitivity of the red cells being used. Variation in titer of more than one dilution in this control disqualified the run. The tests were read very closely and the least perceptible agglutination, when compared with the cell control, was called positive. Agglutination in a dilution of 1 to 16 or greater was considered significant.

Indifferent Streptococcus Agglutination Test (SA).—The method described by Thomas and associates[9] was adhered to in exact detail. A culture of the streptococcus 344 was obtained from Dr. M. D. Eaton. When results were read, only tubes in which there was definite agglutination and clear fluid were called positive. Titers of 1 to 10 or greater were considered significant.

Elementary Body Type Virus Complement Fixation Test (EB).—"Lygranum CF"* was used as antigen and the technique previously described by one of us was followed.[12] The lowest dilution of serum tested was 1 to 10. No single titer, only a change in titer, was considered significant.

Kahn Flocculation Test for Syphilis.—The standard Kahn procedure[18] was followed, but only a single reading, after fifteen minutes, was made. The antigen was furnished by the Army Medical School. Readings which gave a total of from 7 to 12 plus were called positive, those with 2 to 6, doubtful, and less than 2, negative.

Kolmer Complement Fixation Test for Syphilis.—The modified micro-Kolmer test, previously described,[12] was used. The antigen was obtained from the Army Medical School. With the patient's serum diluted 1 to 5, 3 or 4 plus fixation was called positive. If any other fixation was present, the test was reported as doubtful.

Heterophil Test.—The shaking and spinning technique described by Crawford and Hertert[19] was followed. Defibrinated sheep cells, never more than three days old, were used. A titer of 1 to 40 or greater was considered significant.

Weil-Felix Test.—The method of Crawford and Hertert[19] was employed. Antigens were prepared from Proteus OX-2, OX-19, OX-K strains. A titer of 1 to 80 or greater was considered significant.

In the interpretation of these tests only a rise or fall in significant titers was considered positive. If a significant titer was maintained unchanged in all specimens, or if the peak antibody titer was only "doubtfully positive," it was interpreted as a questionable reaction.

RESULTS

In Table I is presented a summary of the results when each of the four tests in the "battery" was applied to serial specimens of serum from patients with primary atypical pneumonia. Many of these specimens were inadequate for more than one of the serologic tests for syphilis. Therefore, in the table, the results of both Kahn and Kolmer are grouped together under the simple heading Serologic Test for Syphilis (STS). A positive or doubtfully positive transitory reaction in either of these tests is accordingly indicated.

TABLE I. INCIDENCE OF REACTIONS IN GROUP OF PATIENTS WITH PRIMARY ATYPICAL PNEUMONIA

TEST	TOTAL NUMBER OF PATIENTS TESTED	NUMBER OF TESTS			POSITIVE IN EACH GROUP (%)
		POSITIVE	DOUBTFUL	NEGATIVE	
Cold agglutinin (CA)	68	43	7	18	63
Indifferent streptococcus agglutinin (SA)	36	17	8	11	47
Elementary body complement fixation (EB)	45	12	4	29	26
Serologic for syphilis (STS)	36	6	6	24	17

Sera from sixty-eight patients were studied by us with the CA test. Of these, forty-three (63 per cent) gave a positive reaction. This result may be compared with the forty-three of ninety-three patients (46 per cent) with primary atypical pneumonia studied by the Commission on Acute Respiratory Diseases of the United States Army[8] who had maximum titers of 1 to 16 or

*E. R. Squibb & Sons, New Brunswick, N. J.

above, and the sixty of seventy-four patients (81 per cent) reported by Meikeljohn[20] who had titers of at least 1 to 20. These differences may reflect the geographic sources of patients and criteria for selection of cases. The patients reported by the Commission were all soldiers admitted to the Station Hospital at Fort Bragg, N. C., and seen by at least one member of the Commission. Those in Meikeljohn's study were mostly civilians and college students from the San Francisco Bay region. They were all selected by him. Our patients were drawn from Army posts in various parts of California, Utah, Nevada, and Arizona, where the original diagnoses were made by many different physicians. However, all reports agree that in a considerable percentage of patients with this syndrome, cold agglutinins do develop during convalescence.

There were thirty-six sets of sera which were also examined by us in the SA test. Of these, seventeen (47 per cent) were positive. Although this is a small series of patients, the results are strikingly similar to those reported by other investigators. Thomas and associates[9] found that fifty-five of 101 (55 per cent) and Meikeljohn and Hanford[21] reported that seventy-three of 156 patients (47 per cent) had titers of at least 1 to 10.

The sera of only twelve of the forty-five patients (26 per cent) we studied with the EB test were positive. The use of the EB test in the study of primary atypical pneumonia has already been discussed by one of us.[12] It probably segregates those instances of the syndrome caused by viruses of the ornithosis-psittacosis group. Since the test does not differentiate between the members of this group, its interpretation should not be carried beyond this general statement. Our "Lygranum CF" antigen crosses serologically with the psittacosis virus, and it is therefore of interest that Rein and Elsberg[14] reported that nineteen of a group of thirty-six patients with primary atypical pneumonia (53 per cent) had positive complement fixation tests for psittacosis.

Thirty-six of our patients had consecutive serologic tests for syphilis performed. Of these, six (17 per cent) had transitory, definitely positive serologic tests for syphilis develop during convalescence. An additional six showed transitory doubtfully positive reactions. Therefore, a total of twelve (33 per cent) in this small series of patients developed some change in the serum globulins which could be detected with the serologic test for syphilis. It should be noted that all of these were, in reality, "false" positive reactions. In 1941 Hegglin[13] reported in the Swiss literature nineteen cases of an atypical pneumonia with repeated strongly positive transitory serologic tests for syphilis and suggested that this was a new syndrome. Recently at the Army Medical School in Washington, D. C., Rein and Elsberg[14] subjected serial specimens of serum from thirty patients with atypical pneumonia to a battery of six serologic tests for syphilis and found that seventeen (24 per cent) had transitory positive reactions in two or more of these tests.

In Table II is presented a summary of the results when the CA, SA, and STS were applied to the "control" sets of sera.

TABLE II. INCIDENCE OF REACTIONS IN GROUP OF CONTROL PATIENTS WITH VARIOUS RESPIRATORY DISEASES

TEST	TOTAL NUMBER OF PATIENTS TESTED	NUMBER OF TESTS			POSITIVE IN EACH GROUP (%)
		POSITIVE	DOUBTFUL	NEGATIVE	
Cold agglutinin (CA)	32	6	0	26	19
Indifferent streptococcus agglutinin (SA)	25	3	3	19	12
Serologic for syphilis (STS)	22	2	6	14	9

The fact that six of thirty-two (19 per cent) of these individuals gave a positive CA test seems strikingly high. However, a review of the clinical diagnoses in these positive cases suggests that some of these may actually have been instances of primary atypical pneumonia which were incorrectly classified due to our arbitrary division of cases. Two of the patients were diagnosed as having "bronchopneumonia," one "pneumonitis," one "lobar pneumonia," one "bronchiectasis," and the last "nasopharyngitis."

Three of the twenty-five patients tested with the SA reaction gave positive results. Diagnosis in one of these was "questionable influenza" and in the other two, "nasopharyngitis."

There were two transitory positive STS tests among the twenty-two sets of sera examined. Both of these were in patients with "nasopharyngitis"; in addition, one had a positive CA and negative SA, while the other had a positive SA and negative CA test.

Although our "control" group is small, it does tend to indicate that a positive CA or SA test in a patient with respiratory disease is strong presumptive evidence in favor of a diagnosis of "primary atypical pneumonia."

Since the CA test was found to have the highest incidence of positive reactions among patients with primary atypical pneumonia, an attempt was made to correlate the results in the SA, EB, and STS tests with those in the CA test. This is shown in Table III. In our limited series it appears that the majority of positive SA tests occurred in individuals who had developed positive CA reactions. This was also Meikeljohn and Hanford's[21] experience. However, most of our positive EB tests were in patients who did not have an increase in cold agglutinins. "False" positive serologic tests for syphilis were found to be unrelated to the distribution of positive CA tests. In Table III is also indicated that the CA and SA tests reflect different antibodies. There were five patients who had positive CA reactions and negative SA tests and four who had positive SA reactions and negative CA tests. Evidence that the EB and STS are not related to each other has been presented elsewhere.[12] These four tests would therefore seem to reflect at least four distinct antibodies which might arise in an individual as a consequence of his having an attack of atypical pneumonia.

TABLE III. DISTRIBUTION OF OTHER REACTIONS IN RELATION TO REACTIVITY IN COLD AGGLUTININ TEST IN PATIENTS WITH PRIMARY ATYPICAL PNEUMONIA

COLD AGGLUTININ	REACTIONS IN OTHER TESTS	INDIFFERENT STREPTOCOCCUS AGGLUTININ (SA)	ELEMENTARY BODY COMPLEMENT FIXATION (EB)	SEROLOGIC TESTS FOR SYPHILIS (STS)
Positive	+	11	4	3
	?	4	3	3
	–	5	20	14
Doubtful	+	2	2	0
	?	0	0	1
	–	2	3	3
Negative	+	4	6	3
	?	4	1	2
	–	4	6	7
Total patients in each test		36	45	36

When the results of these tests are summarized for each patient, as is done in Table IV, patterns of serologic reactivity or "profiles" emerge. However, among twenty patients with primary atypical pneumonia whose serial specimens of serum were examined with this "battery" of tests, fifteen different profiles appeared!

TABLE IV. REPRESENTATIVE REACTIONS OF PATIENTS WITH PRIMARY ATYPICAL PNEUMONIA

PATIENTS	SPEC. NO.	DAYS AFTER ONSET	COLD AGGLUTININ (CA)	INDIFFERENT STREPTOCOCCUS AGGLUTININ (SA)	ELEMENTARY BODY COMPLEMENT FIXATION (EB)	SEROLOGIC TESTS FOR SYPHILIS STS	
						KAHN	KOLMER
1. Primary atypical pneumonia	1424	10	16,384				N
X-ray, +	1454	31	256	<10	<10	N	
W.B.C., 6,000	1464	39	<16	<10	<10	N	N
	Profile		+	−	−		−
2. Primary atypical pneumonia	5096	24 days before onset	<16	<10			
X-ray, +	5106	7	256	40	<10		N
W.B.C., 5,800	5112	35	256	40	<10	N	N
	Profile		+	+	−		−
3. Primary atypical pneumonia	1769	15	256	40	<10	01±	+1
X-ray, +	1781	25	1024	80	<10	N	N
W.B.C., 3,700	1786	43	1024	20	<10	N	N
	Profile		+	+	−		?
4. Primary atypical pneumonia	1691	13	16	80			+4
X-ray, +	1698	23	<16	160	<10		+4
W.B.C., 5,600	1744	54	<16	40	<10	N	N
	Profile		+	+	−		+
5. Primary virus bronchitis	1358	5	<16	10	10	N	N
X-ray, −	1379	16	64		20		+4
W.B.C., 8,400	1401	36	<16	80	<10	N	N
	Profile		+	+	+		+
6. Primary atypical pneumonia	733	18	<16	<10	80	N	N
X-ray, +	804	25	<16	10	80		N
W.B.C., 7,500	964	48	<16	<10	10	N	+4
	Profile		−	+	+		+
7. Primary atypical pneumonia	1346	9	<16	<10	<10	N	+2
X-ray, +	1362	28	<16		20		N
W.B.C., 10,000	1380	41	<16	<10	10	N	N
	1416	71	<16		<10		N
	Profile		−	−	+		?
8. Primary atypical pneumonia	1774	13	<16	<10	<10	N	N
X-ray, +	1783	27	<16	10	<10	N	N
W.B.C., 9,750	1796	41	<16	10	<10	N	N
	Profile		−	+	−		−

A review of our data for the time of appearance of these various antibodies revealed a general conformity to what has been previously reported by others.[7, 14, 21] Although in a few instances the CA and SA were apparent within the first week of illness, the peak of CA usually came between the tenth and twenty-fifth days, while that of SA usually did not appear until after the third week. The EB antibody appeared during the second and third weeks when it also reached its peak. The most frequent time for appearance of reagin as measured by the STS was the second week, although in an occasional patient it was not detected until much later.

Early in our study, seventy-three consecutively received specimens of serum from patients with the diagnosis of primary atypical pneumonia were tested with the CA, heterophil, and Weil-Felix reactions. This was prompted by a report that Cheney and Gardner[16] had found that seven of eight of their patients with atypical pneumonia who were tested had positive Weil-Felix tests. The heterophil test was included, since the clinical picture of infectious mononucleosis, in which there is a high incidence of positive reactions, may resemble atypical pneumonia. In our group of seventy-three specimens there were twenty with significant CA titers. Two had heterophil levels of 1 to 40, and only one had a significant Weil-Felix titer.

DISCUSSION

The development of so many different combinations of the antibodies which are indicated by the CA, SA, EB, and STS in our group of clinically similar cases of pneumonitis suggests again that primary atypical pneumonia is a syndrome which can be produced by several different agents. As indicated by the results of the EB tests, a number of our cases were probably caused by some member of the ornithosis group of viruses. We also know that occasionally the influenza virus, the rickettsia of Q fever,[22, 23] and even the fungus *Coccidioides immitis*[24] may simulate this picture. This is not unlike the familiar difficulty of attempting to recognize clinically the significant differences between the various encephalitides and forms of dysentery.

It would be convenient to think that all the patients who developed positive CA tests represented infections with a single, as yet unknown, etiologic entity. In support of this concept are the facts that it was from this group that Eaton recovered a virus which produced pneumonitis in cotton rats[6] and from which the transmission of the human disease with bacteria-free filtrates was demonstrated.[3] However, it leaves unexplained the appearance of cold agglutinins in an occasional patient with a positive EB test, the not invariable association of cold agglutinins with the development of indifferent streptococcus agglutinins, and the relatively high incidence of false positive STS[15] among patients with this syndrome. It has been suggested that primary atypical pneumonia might be caused by the synergistic action of several agents; for example, a virus and bacterium, as in swine influenza. Although we prefer not to think of a multiplicity of agents as the basis of a single illness, it may offer the only logical explanation for the multiplicity of serologic reactions. On the other hand, Landsteiner[25] has pointed out that "complex materials can give rise to as many antibodies as there are antigenic components." Consequently, a single very complex agent might conceivably be responsible for the more frequently recognized atypical pneumonias and the variety of serologic profiles might represent but another example of individual variation in immunity. Fascinating deductions as to the antigenic composition of this agent follow when the nature of the antigens we used for detecting these antibodies are considered. Thus it has been shown that the active principle in the indifferent streptococcus is a polysaccharide,[26] in the STS it is a cardiolipid,[27] and in the CA test it is contained in the stroma of the human erythrocyte.[28]

Intriguing as this may be, at the present state of our knowledge we should merely note that from 40 to 80 per cent of patients with the syndrome of primary atypical pneumonia develop an increase to significant levels of cold agglutinins; that those who develop CA are usually the ones in whom SA appear, and that the majority of individuals who have positive EB tests do not have positive CA reactions. The relative frequency of so-called "false positive" serologic tests for syphilis in this syndrome is also worthy of note.

SUMMARY

1. Serial specimens of serum from sixty-eight patients with a clinical diagnosis of primary atypical pneumonia were examined in one or more of a "battery" of serologic tests. The tests employed were the cold agglutinin (CA), indifferent streptococcus agglutinin (SA), elementary body type virus complement fixation (EB), and the Kahn flocculation and Kolmer complement fixation tests for syphilis (STS).

2. There were 63 per cent of these patients who were found to have positive CA tests, 47 per cent who developed positive SA reactions, 26 per cent with positive EB tests, and 17 per cent with a transitory positive STS. These results were significantly different from those found in 32 "control" sets of sera.

3. It was demonstrated that a positive SA test is more likely to occur in a patient with a positive CA reaction, that the majority of individuals with positive EB tests do not have positive CA tests, and that there is no apparent relationship between the occurrence of any of these reactions and a transitory positive STS.

4. In a group of seventy-three consecutively received sera from patients with primary atypical pneumonia, only two significantly high heterophil titers and one Weil-Felix titer were found.

5. The frequency with which CA, SA, EB, and STS antibodies are found to develop in patients convalescing from primary atypical pneumonia suggests an immunologic association with the etiologic agent (or agents) of the disease. The many combinations of response which are measured by the tests reported here may be a reflection of individual variation or of several different agents producing the same syndrome. Until the etiologic agent for the majority of instances of primary atypical or viroid pneumonia is identified, these tests serve the practical purpose of more clearly defining the disease.

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PHAGOCYTIC ACTIVITY OF NEUTROPHILES IN ANEMIAS

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FOR years students of anemia have pondered over the physiologic mechanisms that enable the severely anemic patient with white cell counts of from 1,500 to 3,000 per cubic millimeter to avoid overwhelming infections. The question has also been raised as to how these patients are able to walk about and maintain a mild activity with red cell counts around a million and hemoglobin values of from 20 to 30 per cent. As yet there is no complete answer for either of these problems, but it appears that we have found at least a partial explanation for the anemic patient's defense against infections. In studying the phagocytic activity of neutrophiles in malnourished patients seen at the Nutrition Clinic of Hillman Hospital in Birmingham, we observed in one of our most poorly nourished patients, who also had a macrocytic hyperchromic anemia, a greatly increased phagocytosis. As more patients with anemia were tested we discovered that without exception patients with either macrocytic hyperchromic or microcytic hypochromic anemia had an increased white cell activity and that the increase in phagocytosis was roughly proportional to the severity of the anemia.

METHODS

Phagocytic activity was measured on whole blood according to the method of Cottingham and Mills.[1] Briefly, this consists of diluting in a paraffined serologic tube 0.5 c.c. of venous blood with 0.5 c.c. heparinized† saline; 0.2 c.c.

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of a standardized suspension of *micrococcus candidus* is added to the tube, which is quickly gassed with a mixture of 95 per cent O_2 and 5 per cent CO_2, stoppered, inverted twice, and agitated in a water bath at 38° C. for four minutes. Films are made immediately and then stained. A differential white cell count is made, and at the same time the number of bacteria in 100 neutrophiles is counted and averaged. The figure obtained in this way is arbitrarily taken as the phagocytic activity of the blood. The minimum error of the method when control values from day to day are compared (see Table I, column 4) is approximately ±10 per cent. The principal variable is the density of the bacterial suspension. This was standardized by means of an Evelyn colorimeter using a 515 $\mu\mu$ filter. Variations of from 2 to 3 scale divisions on the galvanometer produced changes in phagocytic counts of from 30 to 40 per cent. Another source of error appears as the average number of bacteria per neutrophile approaches 30. Since it is impossible to count accurately more than 30 bacteria within a cell, this is then taken as the largest number used in determining the phagocytic activity. If there is a relatively large number of leucocytes "filled" with bacteria, the average then becomes more inaccurate. Hence the error in such cases is probably at least from 20 to 30 per cent.

All of the results are based upon a comparison of the phagocytic activity of leucocytes of anemic patients with those of "normal" persons. The blood of one of us (L. J. B.) was used as the control, since he is free from any disease, consumes an excellent diet rich in proteins, minerals, and vitamins, and has always had normal blood during occasional tests over the past two years and frequent tests during the past five months. The white blood cell total has varied between 7,700 and 9,800, with a differential count always within normal limits of variation. The red cell total and hemoglobin have varied between 4.89 and 5.10 millions and 14.7 and 15.9 Gm., respectively. The phagocytic activity of this control blood checked within 10 to 15 per cent the phagocytic activity of the blood of other workers in the laboratory who are of both sexes and are in equally good health. One such worker (R. B. J.) was used as the "normal" on some days.

Each day an experimental test was made on the blood of anemic patients, "normal" blood was collected and treated identically. Variations due to changes in the density of the bacterial suspensions from one day to the next should give comparable results between the "experimental" and control blood samples. Thus in order to eliminate the day-to-day differences in phagocytic activity arising from the method itself, the value obtained for the blood of the anemic patient is divided by the value obtained for "normal" blood on the same day. This gives a calculated value which is designated as "per cent 'normal' phagocytosis."

RESULTS

The results from twenty-nine patients are shown in Table I. The phagocytic activity of the patient is given in column 3, while the "normal" phagocytic activity on the same date is shown in column 4. The five values marked with the asterisk were obtained with R. B. J. as the control. The per cent "normal" (numbers in column 3 divided by those in column 4 × 100) shown in column 5 is more than 100 per cent in all cases. When the magnitude of these values is compared with the red cell counts (column 6) and hemoglobin level (column 7), it can be seen that in general the more severe the anemia, the higher the phagocytosis. The mean per cent "normal" phagocytosis in those

TABLE I

(1) CASE	(2) DATE	(3) PHAGOCYTIC ACTIVITY	(4) "NORMAL" PHAGOCYTIC ACTIVITY	(5) PER CENT "NORMAL"	(6) R.B.C. (MILLIONS)	(7) HG. (GM.)	(8) W.B.C.	(9) PER CENT (P. M. N.)	(10) TYPE ANEMIA
1	5/3	22.38	9.93	225	1.74	7.8	5,300	51	Macrocytic hyperchromic
2	4/10	26.80	9.49	283	1.35	5.7	1,600	62	Macrocytic hyperchromic
3	6/27	21.81	8.50	257	2.43	7.8	3,850	55	Macrocytic hyperchromic
4	5/3	21.55	9.93	216	2.07	7.9	6,200	56	Macrocytic hyperchromic
5	6/25	15.70	8.80	178	2.54	11.6	3,500	62	Macrocytic hyperchromic
6	5/14	13.93	9.86	141	2.70	12.0	6,050	63	Macrocytic hyperchromic
7	6/27	11.88	8.50	140	2.63	10.1	7,400	62	Macrocytic hyperchromic
8	5/16	15.01	9.90	152	1.94	8.6	5,450	69	Macrocytic hyperchromic
9	4/30	9.94	8.14	122	4.47	8.0	7,900	72	Microcytic hypochromic
10	4/21	13.97	10.41	134	2.64	9.1	4,050	72	Macrocytic normochromic
11	5/2	20.51	11.08*	185	2.69	10.4	4,950	67	Macrocytic hyperchromic
12	5/2	20.17	11.08*	182	3.52	10.7	5,950	45	Macrocytic normochromic
13	5/17	17.81	7.14*	250	2.27	8.8	5,300	65	Macrocytic hyperchromic
14	5/17	24.24	7.14*	343	1.60	5.3	2,300	41	Macrocytic hyperchromic
15	5/23	17.39	10.10	172	3.69	8.3	6,900	49	Microcytic hypochromic
16	6/19	11.81	9.86	119	2.00	9.2	7,350	74	Macrocytic hyperchromic
17	6/15	13.06	8.91	148	2.52	6.9	6,500	85	Microcytic hypochromic, renal insufficiency, chronic G.U. infection
18	4/25	13.27	8.99*	148	4.30	8.6	8,100	73	Microcytic hypochromic
19	4/26	26.45	9.85	269	1.02	6.5	2,100	53	Macrocytic hyperchromic
20	5/14	17.54	9.86	178	3.15	10.7	3,000	64	Macrocytic hyperchromic
21	5/3	20.32	9.93	204	1.85	8.6	4,500	44	Macrocytic hyperchromic
22	5/14,	22.78	9.86	231	3.19	8.6	----	34	Microcytic hypochromic
23	6/1	13.60	10.39	131	2.76	11.2	4,450	73	Macrocytic hyperchromic
24	4/17	16.35	11.67	153	1.11	4.6	6,150	75	Macrocytic hyperchromic
25	3/24	21.36	9.19	232	2.56	9.1	5,450	51	Macrocytic hyperchromic
26	4/26	19.38	9.85	197	0.79	3.3	3,250	49	Macrocytic hyperchromic
27	4/21	15.42	10.41	148	4.11	10.1	4,300	62	Macrocytic hyperchromic
28	5/23	14.73	10.10	146	4.20	6.5	6,500	30	Microcytic hypochromic
29	5/23	16.19	10.10	160	2.78	4.9	6,150	48	Microcytic hypochromic

*Control R. B. J. All other controls L. J. B.

cases where red cell counts are under two million is 228 per cent. As the erythrocyte counts rise to between 2 and 3 million and to above 3 million, the mean falls to 176 per cent and 166 per cent, respectively. A similar comparison between the per cent "normal" phagocytosis and the total number of leucocytes (column 8) fails to show as close a correlation, but the three most leucopenic patients (Cases 2, 14, and 19) have very high phagocytic activities. Moreover, when the total number of neutrophiles alone is considered (found as the product of columns 8 and 9), the inverse correlation is more apparent. However, as explained below, there are exceptions to a high phagocytic activity accompanying low white cell counts.

Cases 14, 18, 25, 26, and 29 of Table I deserve special consideration since they show quite dramatically the decrease in per cent "normal" phagocytosis with remission of the anemia (Table II). In fact, the phagocytic activity *falls below normal* in each, except Case 14, when the red cells are approximately normal. These subnormal values are not unexpected since it has been shown[1, 2] that phagocytosis is depressed in experimental animals on deficient diets. Observations[3, 4] on malnourished patients without anemia show also that the phagocytic activity is less in such persons than in "normals." Thus, as the malnourished patients with anemia improve with respect to their anemia, the phagocytic count may become lower than "normal."

TABLE II

(1) CASE	(2) DATE	(3) PER CENT "NORMAL"	(4) R.B.C.	(5) HG.	(6) W.B.C.
14	5/17	343	1.60	5.3	2,300
	6/20	152	3.24	9.7	5,300
	7/11	151	3.80	10.7	6,300
18*	4/25	148	4.30	8.6	8,100
	4/30	131	4.50	9.4	8,400
	5/19	60	4.92	12.5	6,700
	6/23	38	4.97	14.0	6,000
24	4/17	153	1.11	4.6	6,150
	5/ 2	115	2.90	8.3	5,150
	5/21	72	3.37	8.8	3,550
25†	4/14	171	3.14	10.1	4,400
	4/28	333	1.87	7.6	3,050
	5/24	270	1.82	6.4	5,650
	6/19	51	3.32	11.1	9,250
26	4/26	197	0.79	3.3	3,250
	5/17	288	0.93	4.4	4,150
	5/24	350	1.38	7.0	4,450
	6/23	108	3.22	11.7	4,600
	7/14	51	4.57	12.7	----
29	5/ 2	193	2.82	5.5	3,200
	5/23	160	2.78	4.9	6,150
	6/19	97	4.26	10.9	6,400
	7/11	77	4.18	12.8	----

*See Fig. 1.
†See Fig. 2.

The changes in the number of white blood cells accompanying the red cell changes are shown in Table II, column 6. In Cases 14, 25, 26, and 29 there is an increase in white blood cell counts as the per cent normal phagocytosis decreases, but in Cases 18 and 24 there is a decrease in leucocyte counts. Thus the correlation between total leucocytes and phagocytic activity does not always hold, whereas no exceptions have as yet been found in the correlation between the degree of anemia and phagocytosis.

Case 26 should be especially noted, for within a period of twenty-eight days (4/26 to 5/24) the phagocytic activity increased from 197 per cent to 350 per cent. During this interval the erythrocyte count rose from 0.79 to only 1.38 millions and the hemoglobin changed from 3.3 to 7.0 Gm. Could it be possible that over a long period with such severe anemia the phagocytic activity increases and provides additional protection from infection? Could not the time factor be important in determining the phagocytic count? In this case, as well as in all others, the remission of the anemia (5/24 to 6/23) was accompanied by an approximate return to white cell activity to normal (or below).

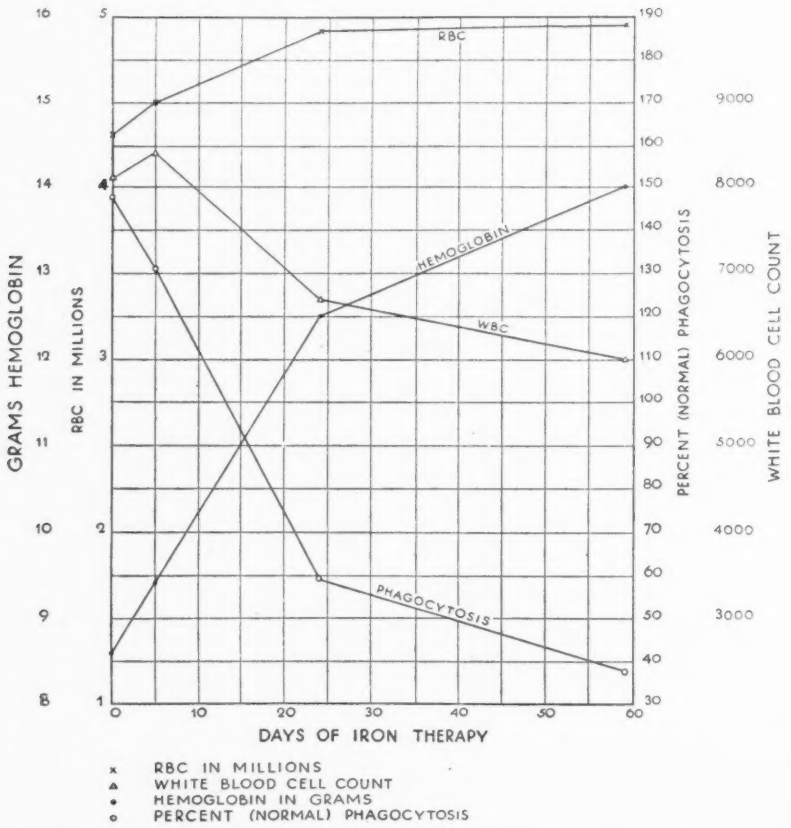

Fig. 1.—Decrease in phagocytosis with therapeutically induced remission in a patient with hypochromic anemia.

The results for Case 18 shown in Table II are presented graphically in Fig. 1. Iron therapy* was initiated the day the first counts were made and was continued throughout the fifty-nine days covered by the graph. Fig. 1 demonstrates in a striking way that the change in total phagocytosis of the neutrophiles is inversely proportional to the change in hemoglobin content of the blood. The two curves in this case are perfect mirror images of one another, while the total erythrocyte curve changes but slightly. The total leucocyte count varies within normal limits and is independent of the other factors shown.

The data of Case 25 are plotted in Fig. 2. The initial drop in erythrocytes, hemoglobin, and leucocytes is accompanied by an approximate twofold in-

*White's "Mol-Iron" capsules were given three per day. Each tablet contains 3 gr. $FeSO_4$ and 1/20 gr. molybdenum.

crease in phagocytosis. Following the administration of one unit of reticulogen per day from the twenty-ninth through the thirty-ninth day, there was a rapid rise in total red and white cells and in hemoglobin, with a marked fall in phagocytosis occurring during the same interval. There is no mirror image between any pair of curves corresponding to that seen in Fig. 1, but in this case both erythrocytes and hemoglobin were undergoing rapid changes. The white blood cells varied inversely with phagocytosis but not symmetrically.

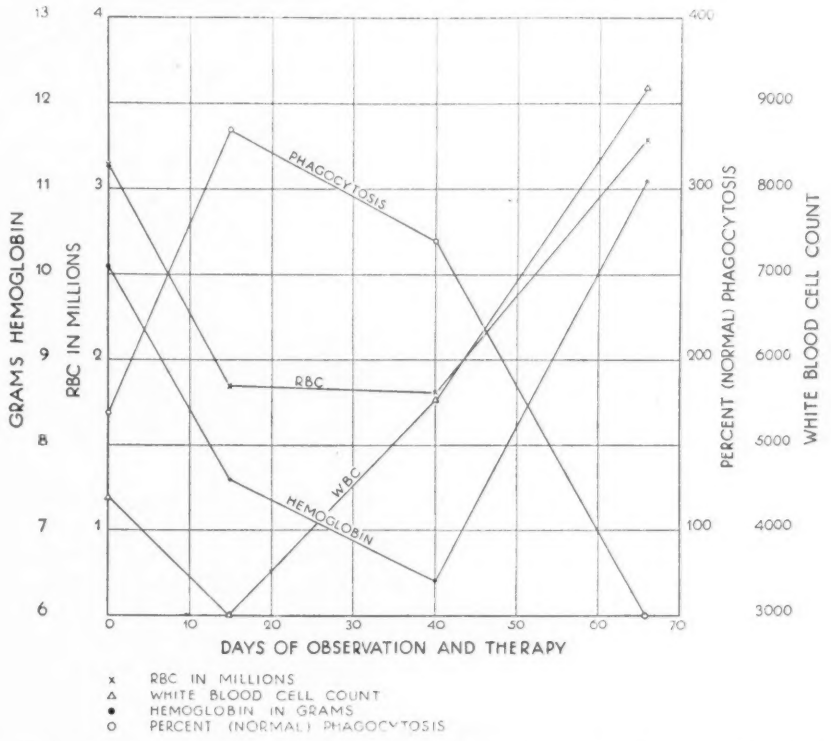

Fig. 2.—Decrease in phagocytosis with therapeutically induced remission in a patient with macrocytic anemia.

DISCUSSION

The resistance of all mammals to infections depends upon several factors. These are usually listed as (1) a structural barrier against invasion, (2) humoral defense mechanisms, and (3) phagocytic cells. The leucocytes are probably the final defense against infection, for the ultimate destruction of bacteria is usually accomplished by the leucocytes. Thus, the total effectiveness of the phagocytic cells rests not only upon their number, but also on the activity of each cell. It is therefore important to observe that the phagocytic activity of the neutrophiles in anemic persons may undergo as much as a threefold increase. This increase occurs at a time when the total number of white cells usually decreases to a value from one-half to one-third normal. Thus, assuming that the product of the total number of neutrophiles and the number of bacteria engulfed per neutrophile reflects the true total phagocytic effort, there is no total loss in the ability of the white cells to overcome the bacteria. Only Case 24 seems to show any significant decrease in the white cell defense mechanism after remission of the anemia, and this may be due primarily to the atypical fall in total leucocytes, for which we have no explanation.

The physiologic basis for the enhancement of phagocytic activity in anemic persons is unknown. At present, experiments designed to provide answers to many questions that naturally arise are in progress. These results will be reported later.

SUMMARY

1. In twenty-nine cases of anemia it has been found that the phagocytic activity is from 119 to 340 per cent of "normal." The magnitude of this activity is roughly proportional to the severity of the anemia but fails to follow the leucocyte count in all cases.

2. As the anemia remits, the phagocytosis decreases and may even fall below "normal" values as the red cells approach normal. In the majority of cases there is an accompanying increase in white cells.

3. These studies, which suggest that in anemia a protective mechanism is set up against infections, may offer an explanation for the rather surprising resistance to infections commonly seen in some persons with anemia.

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FATAL SECONDARY, TOXIC THROMBOCYTOPENIC PURPURA DUE TO SODIUM SALICYLATE

Report of a Case

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INTRODUCTION

IT IS not surprising that the number of substances responsible for the production of secondary thrombocytopenic purpura is an ever-increasing one due to the discovery of new drugs and chemical agents. The wartime diversion of silk and nylon for military purposes, for instance, was followed by the introduction of "leg-stocking color" cosmetic (Sloan[11]), with the result that a new thrombocytopenogenic substance appeared. In contrast, it is extremely surprising to find an old, well-established, and widely used drug like sodium salicylate among the list of known offenders. Those substances which have been definitely incriminated include sedormid, various sulfonamides, organic arsenicals and bismarsen, gold, quinine derivatives, benzol, smallpox vaccine, chenapodium, ergot, dinitrophenol, iodine, hair dye, snake venom, mercury bichloride, colchicine (Custer,[2] Wintrobe,[12] and Limarzi[8]).

Recently two cases of hemorrhagic complications with death following salicylate therapy were reported by Ashworth and McKemie.[1] In both in-

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stances, the predominant change consisted of multiple ecchymoses and hemorrhages throughout the body and especially in the brain. Other than several routine determinations of the red blood count, white blood count, and differential count, no hematologic examinations were performed. Diagnostic procedures usually carried out in hemorrhagic diseases, such as the determination of the clot retraction, bleeding and clotting times, prothrombin time, platelet count, performance of bone marrow examination, and tourniquet test were omitted. In the absence of such data, the causative mechanism of the hemorrhagic phenomena in these cases is completely unclarified.

Nonetheless, on the basis of experimental reports of the occurrence of hypoprothrombinemia during salicylate treatment, these authors conclude that the hemorrhages could be explained on the basis of capillary damage and hypoprothrombinemia. This conclusion is untenable and unwarranted by the evidence which they present.

Search of the literature by the Research Division of the Army Medical Library, personal communications from Dr. Charles A. Doan and Major Richard P. Custer, M.C., and reference to the works of Wintrobe,[12] Kracke,[6] Rosenthal,[10] Limarzi,[8] and Piney and Hamilton-Paterson[9] have failed to reveal a single previously reported case of secondary thrombocytopenic purpura due to sodium salicylate. It is believed that the following case report represents a proved instance of fatal secondary thrombocytopenic purpura caused by sensitization to this drug.

CASE REPORT

On Jan. 26, 1944, while overseas, a 35-year-old Negro soldier was admitted to a field hospital with the complaint that the wrists, shoulders, back, and knees were painful and that the wrists had been swollen for about one week. The temperature had been normal and the patient had no symptoms referable to the cardiovascular or genitourinary systems. Joint pains were so severe that he couldn't "even cock his rifle." On physical examination, swelling of both wrists, fusiform swelling of the finger joints, and fluctuation of both knees could be determined. The heart was essentially negative. Roentgenologic examination showed no evidence of bone or joint changes. The sedimentation rate was 24 mm. at the end of one hour. On the basis of these findings, a diagnosis of rheumatoid arthritis was made. No record is made of any medication.

Five days later, Jan. 31, 1944, the patient was evacuated by a British hospital ship to a numbered general hospital. While on board, further studies revealed a sedimentation rate of 24 mm. per hour; white blood cells, 5,800 per cubic millimeter, neutrophilic granulocytes, 65 per cent; lymphocytes, 33 per cent; monocytes, 1 per cent; and eosinophiles, 1 per cent. Urinalysis disclosed a specific gravity of 1,030, acid reaction, 1 plus albumin, and no sugar. A few pus and occasional red blood cells were found, but crystals or casts were not noted. Physical examination corroborated the findings previously described. Heart, lungs, tonsils, and throat were normal. The gingiva, however, was quite infected. At this time, a history of typhoid disease in childhood and a neisserian infection ten years previously was obtained. The diagnosis of multiple arthritis was concurred in by the English medical staff. Sodium salicylate therapy was initiated, a mixture of natrium salicylicum of unknown amount being given every four hours during the ensuing three days on the boat.

Upon arrival at the numbered general hospital Feb. 3, 1944, the patient continued to complain of pain in both wrists, knees, and in all finger joints. The wrist and finger joints were somewhat tender and swollen, but apparently they showed some improvement over their condition at the onset of the disease. Pain was elicited on movement. Temperature elevations of 100.0 to 100.5° F. were noted every afternoon. The sedimentation rate ranged between 22 to 26 mm., the red blood count was 4,090,000 cells per cubic millimeter, and hemoglobin, 75 per cent. Roentgenologic examination of the affected joints disclosed no evidence of bone or joint disease but did demonstrate some osteoporosis. Studies for malaria and sickling were reported as negative. Search for dental foci of infection was fruitless, although several carious teeth were found and treated. The patient was started on sodium salicylate February 4 and received 80 gr. daily until March 8, attaining a total of 2,640 gr.

in thirty-three days. On that date he was transferred to a transport for evacuation to the Zone of the Interior (U. S. A.) and was en route until March 22, when he was admitted to Fletcher General Hospital.

At this time it is important to point out that none of the formal medical records describing the history, physical findings, diagnoses, or medication arrived with the patient, nor were they available during his entire period of hospitalization. After death and subsequent autopsy, however, we entertained considerable curiosity concerning the etiology of the disease and accordingly requested the Adjutant General's Office to forward to us all the military medical records of this soldier. It was only after their receipt that the previously described formal medical record and history of medication with sodium salicylate was obtained.

On admission the patient was unable to give more than a vague description of his joint pains and their onset. Although closely questioned, he had no information to offer concerning the results of the laboratory and roentgenologic examinations. He stated that he had received "pills" in the other hospitals but that he did not know "what they were." He had received no medication on the boat and had continued to experience similar joint pains. He had had two severe chills and knew that his temperature had ranged to 101 and 102° F. daily.

Physical examination disclosed a somewhat emaciated Negro man, aged 35 years, weighing 120 pounds, and measuring 68 inches. Temperature was 102.2° F.; pulse, 100; blood pressure, 100/60; and respiration, 20. Head, neck, eyes, thorax, lungs, and cardiovascular system were essentially normal. No masses, pain, or tenderness could be determined on palpation of the abdomen. The genitourinary and glandular systems disclosed no abnormalities. The shoulder, elbow, and wrist joints, bilaterally, and all interphalangeal joints were extremely swollen, hot, tender to palpation, and painful on passive and active movement. Shortly after admission to the ward, the patient experienced a severe shaking chill.

Urinalysis was essentially negative; the sedimentation rate was 15 mm. per hour; red blood count was 4,180,000; white blood count, 3,000; hemoglobin, 13 Gm.; granulocytes, 63 per cent; and lymphocytes, 37 per cent. The Kahn test and malaria smears were negative. X-ray examination of chest and heart showed no abnormalities. The working diagnosis was possible rheumatic fever or rheumatoid arthritis.

Sodium salicylate medication consisting of 30 gr. daily in conjunction with sodium bicarbonate was started March 22, 1944. Under this therapy the subjective complaints and objective articular findings showed some improvement, although the temperature remained elevated. On March 26 the patient received one multivitamin capsule three times daily. No other drug was given the patient.

On March 31, nine days following the institution of sodium salicylate therapy, the patient began to experience mild epistaxis. Rhinoscopy disclosed many small petechiae of the nasal mucosa. On April 2, two days later, all medication was stopped. A platelet count was 70,000 per cubic millimeter, clotting time was 5 minutes, 45 seconds; bleeding time was 2 minutes, 15 seconds. In ten days the patient received a total of 300 gr. of sodium salicylate.

On April 3, four days after onset of epistaxis, the patient began to complain of pain in his left flank and commenced to show hematuria of ever-increasing degree. At this time ecchymoses were present in the oral and conjunctival mucosae, but none were seen in the skin. The patient's condition rapidly became worse; nausea and vomiting of gross blood occurred and he voided dark, bloody urine containing clots. There was exquisite pain and tenderness in the lower abdomen, especially over the symphysis. In Fig. 1 are shown the results of hematologic examinations at this time. The tourniquet test was strongly positive.

The clinical and laboratory evidence seemed diagnostic of thrombocytopenic purpura. Sodium salicylate as a causative, toxic agent responsible for the onset of this disease was considered but could not be proved in the absence of evidence of previous medication and sensitization, since, as previously noted, the history of antecedent medication was not at hand during the lifetime of the patient. We were fortunate at this time in having Brigadier General Hugh J. Morgan, M.C., Consultant in Medicine in the Office of The Surgeon General, and Colonel Walter B. Martin, M.C., Consultant in Medicine in the Fifth Service Command, in the hospital on an inspection visit. They concurred in the diagnosis of thrombocytopenic purpura and also considered sodium salicylate as a possible etiologic factor, conceding, however, that on the basis of the available evidence, this hypothesis could not be substantiated.

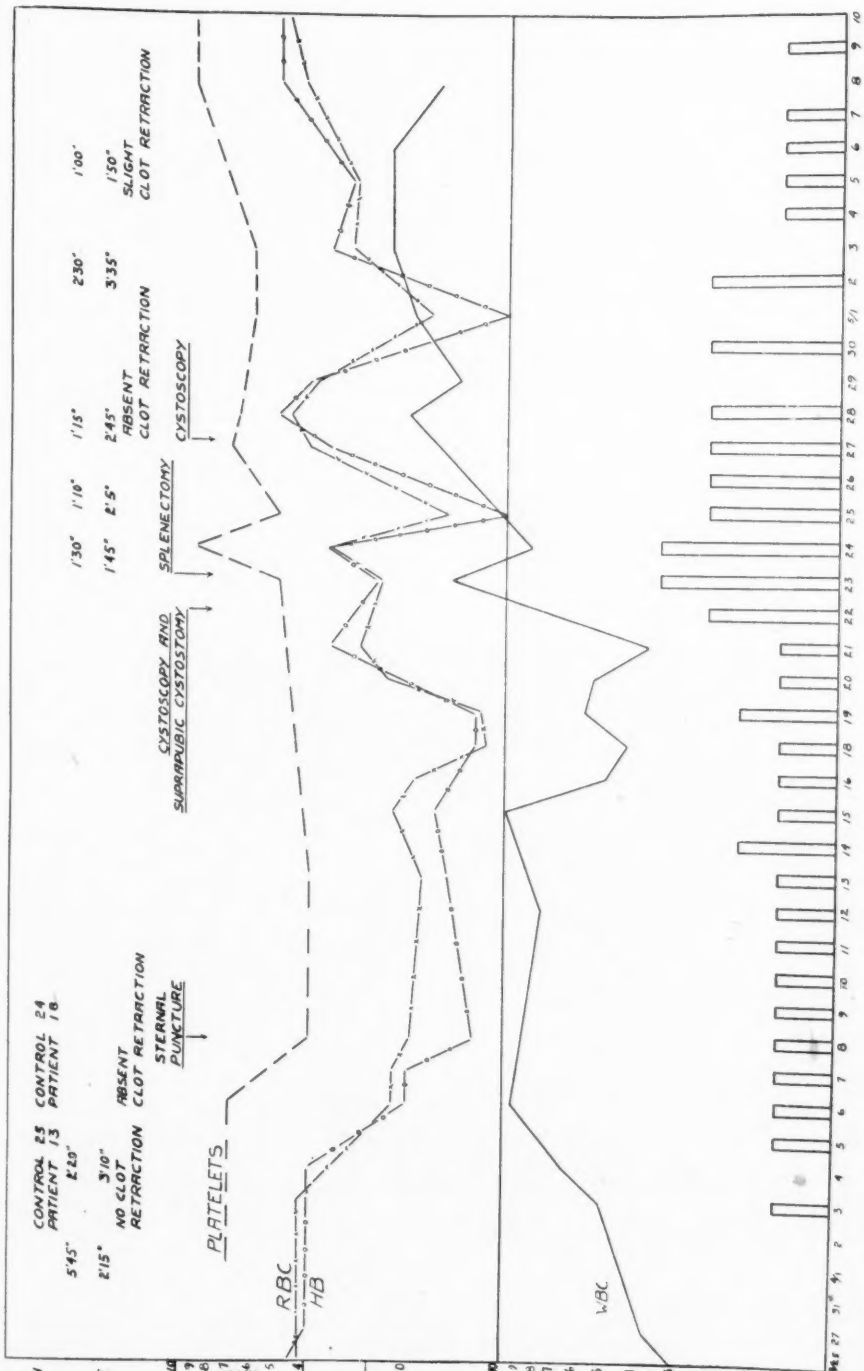

Fig. 1.—This chart shows the consistently low platelet count and absent clot retraction associated with normal clotting and bleeding times. It indicates the progressive, profound anemia and the daily massive transfusions given in the attempt to equalize the hemorrhage. Also illustrated is the absence of thrombocytosis following splenectomy and the leucocytosis resulting from peritonitis. This chart is on a semi-logarithmic scale.

Course in Hospital.—The results of serial hematologic examinations are shown in Fig. 1. Also indicated are the daily massive transfusions necessary to combat the severe hematuria, which, on several occasions, threatened to exsanguinate the patient. The platelet level remained consistently low, never attaining values over 90,000 per cubic millimeter.

In addition to transfusions, vitamin B, vitamin C, calcium gluconate, vitamin K, penicillin, snake venom, reticulogen, and even pentnucleotide were utilized without in any way affecting the course of the disease. Blood cultures, serum agglutination tests for typhoid and paratyphoid fever, undulant fever, tularemia, and typhus, and the heterophile agglutination test were negative. On April 16, .2 c.c. of snake venom was injected intradermally and gave a strongly positive reaction.

On April 8 a sternal puncture was performed. This showed a cellular marrow in which no quantitative or qualitative abnormalities of the erythropoietic and myelopoietic elements could be found. Megakaryocytes appeared plentiful but demonstrated qualitative alterations, such as vacuolization and hyalinization of the cytoplasm, absence of granulation, and pyknosis of the nuclei.

The patient's hematuria, dysuria, frequency, and difficulty in voiding increased steadily. On April 21 a cystogram was taken which disclosed six sharply demarcated, spherical, negative shadows both on the right and on the left, averaging about 2 cm. in diameter (Fig. 2), which were interpreted as bladder tumors of unknown etiology. On the basis of the unusual roentgenogram, and because it was thought that the hemorrhage might be arising from the tumors, cystoscopy was undertaken the following day. In addition to multiple areas of submucous hematomas, two spherical neoplasms were found. They appeared to be sessile and solid and showed bullous edema on their surfaces. It was felt that the bleeding was coming from one of the tumors, although the exact site of hemorrhage could not be seen. The lesions were regarded as rare bladder tumors and their removal was advised.

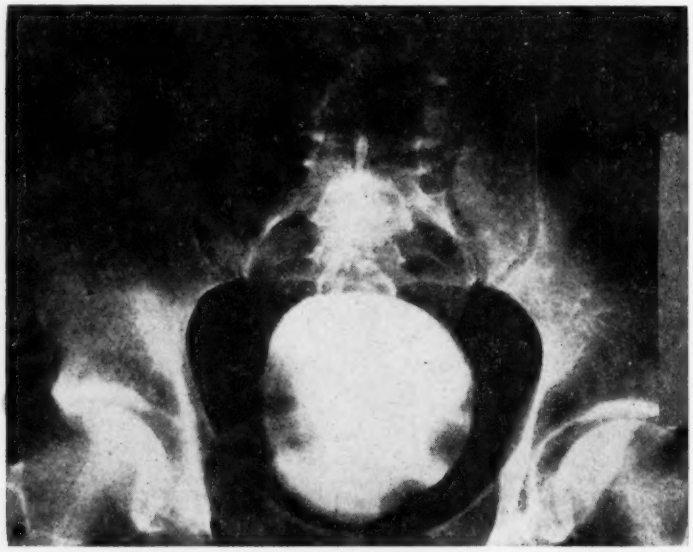

Fig. 2.—Cystogram showing the filling defects caused by multiple, smooth-surfaced, round tumor masses.

Accordingly, a suprapubic cystotomy was performed that day. The bladder was found to be thick-walled and edematous. There were multiple tumorlike projections of the mucosa on both sides of the bladder and several were removed by high frequency current. On cut section they consisted of large, firm, submucous hematomas. Control of the bleeding was only partially successful by electrocoagulation.

On the following day the patient's condition was extremely precarious and the advisability of performing a splenectomy was considered. As an emergency lifesaving measure, therefore, the operation was performed. The results of the histologic examination of the spleen will be considered in connection with the autopsy findings.

There ensued a temporary improvement in the urinary hemorrage and in the hematologic status, but this was short lived, to be followed by definite signs of peritonitis which

appeared in the region of the cystotomy and which gradually became diffuse. Distention, cardiac embarrassment, and pulmonary involvement became exceedingly distressing; the temperature and white blood count rose steadily and the patient died May 13, 1944.

Autopsy Protocol

Autopsy revealed a 35-year-old emaciated Negro man. The skin of the palms and soles and the conjunctival, oral, and nasal mucosa were studded with multiple petechiae. The abdomen was dull to percussion in the flanks and a fluid wave could readily be elicited. Generalized lymphadenopathy was absent.

On incision, the pectoral and abdominal muscles and the fascial spaces contained a few circumscribed areas of hemorrhage. The peritoneal cavity contained 9½ liters of thick, dirty, gray-red, viscid fluid. There was a diffuse plastic fibrinopurulent hemorrhagic peritonitis present throughout the abdominal cavity, lining the visceral as well as the parietal peritoneum and matting loops of large and small intestine together. The dome of the liver and corresponding diaphragmatic surfaces were completely covered by this shaggy exudate. The posterior wall of the bladder was densely adherent to loops of intestine, creating many loculated fluid-filled cavities. The liver was small and the anterior edge was thin and wrinkled.

The pericardium and pleural cavities showed moderate effusion. The linings were studded with ecchymoses.

The lungs revealed considerable subpleural and parenchymal hemorrhages. Congestion and edema were prominent.

The heart, aorta, pancreas, and adrenals disclosed no unusual changes.

The anterior edge of the liver was wrinkled and atrophic and the consistency was very soft. The capsule of Glisson was lined by a thick, fibrinohemorrhagic plastic exudate, the surface of which was shaggy and white-gray, while the deeper portions were red, dark, and firm. On cut section the liver parenchyma was moist and of grass green-yellow hue. The lobular markings were not distinct. In several places small hemorrhagic droplike foci were noted.

Many hemorrhages were seen throughout the wall of the gastrointestinal tract. The serosa was lined by a similar plastic exudate. Many large, soft, swollen lymph nodes were present in the radix mesenterii and especially in the para-aortic area at the bifuraction.

Fig. 3.

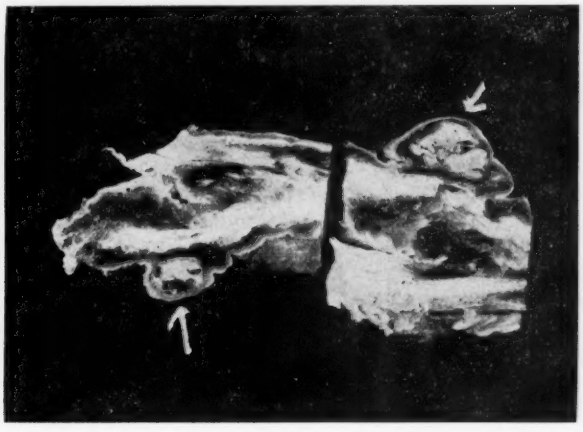

Fig. 4.

Fig. 3.—Surface of bladder showing the marked discoloration of the mucosa and the presence of a polypoid tumor.
Fig. 4.—Cross section of the bladder wall showing the thickening and hemorrhagic discoloration of all coats. Arrows indicate the submucous hematomas.

The kidneys showed no gross hemorrhages. The bladder was removed with the skin and abdominal wall to which it was sutured in toto. The mucosa was dark, discolored, swollen, and nodular (Fig. 3). Lateral to the left ureteral orifice were noted two cherry-sized, smooth-surfaced, dark discolored, soft masses. Lateral to the right orifice, a rough, shaggy, sutured substance defect of the mucosa and the bladder wall could be seen, the surface of which was lined by yellow-gray, necrotic, friable material. Cut sections through the tumor masses revealed large, formed, soft clots in the submucosa which raised the

epithelial lining above the surface (Fig. 4). The entire wall of the organ was swollen, dark, discolored, and hemorrhagic.

The head and brain were not examined.

The spleen was removed twenty days ante mortem. It weighed 115 grams and measured 10 by 7 cm. The capsule was smooth and tense. On section it was dark and of firm consistency and the pulp scraped with difficulty. The Malpighian corpuscles were hardly noticeable.

Histologic Examination of Tissues

Lungs.—Microscopic examination of lungs revealed the presence of many variable-sized hemorrhages throughout the organ, associated with atelectasis and congestion.

Heart.—The heart showed no unusual changes.

Fig. 5.—Photomicrograph of liver demonstrating the thick plastic exudate of the capsule and the severe toxic necrosis (×112).

Liver (Fig. 5).—The capsule of Glisson was thickened and covered with a layer of organized fibrinohemorrhagic exudate. The subcapsular tissue showed a diffuse round cell and fibroblast accumulation. The liver parenchyma was studded haphazardly by many areas of fresh and old hemorrhages. Some of these were mere punctate foci, while others were quite extended and appeared to be both centrolobular and peripheral. Many hemorrhagic foci showed beginning fibroblastic organization, while others, however, were composed of fresh blood. The liver cells were granular, swollen, and vacular and contained a considerable amount of light yellow-brown pigment. Many of the bile canaliculi were distended with firm, clumping, bile pigment thrombi. There were areas of necrobiosis characterized by the presence of fragments of liver cords and cells, many of which possessed highly pyknotic nuclei. There was a diffuse acute and chronic inflammatory reaction comprising round and plasma cells and granulocytes. The sinusoids were congested, particularly in the mid-zones. The Küpfer and endothelial cells showed a very prominent swelling. Many of the swollen endothelium were lying free within the lumen and were packed with pigment. Sections from various portions of the liver revealed necrotic and hemorrhagic changes ranging from minimal to extensive.

Pancreas.—The pancreas was normal.

Spleen.—The capsule was intact and of uniform thickness. The trabeculae were thick and fibrous. There was marked reduction in the number and size of the Malpighian bodies, only a few normal nodules being present. The usual lymph follicle was small and possessed a rudimentary germinal center. Its cells were markedly swollen and irregular in size; the nuclei stained poorly and were vesicular in appearance, and the cytoplasm was quite acidophilic. Many small, basophilic particles derived from degenerated pyknotic nuclei

were lying within these swollen reticulum cells. There appeared to be considerable midfollicular fibrinous exudate (toxic reaction).

The sinusoids were markedly congested. The stroma was dense and filled with solid sheets of large, swollen plasma and reticulum cells. These possessed large, vesicular, lobulated or reniform nuclei which appeared to be somewhat eccentrically located within the cell. The cytoplasm was usually moderately or strongly acidophilic. Large numbers contained a dark brown or yellow, clumpy or granular pigment. Phagocytosis of basophilic particles which might possibly have been derived from necrotic cells, platelets, or extruded erythrocytic nuclei was noteworthy. There was considerable thickening of the walls of the small terminal arterioles. In many cases the lumens were markedly narrowed and, in a few instances, completely occluded.

In occasional sections, areas of coagulation necrosis were present. These foci were diffusely dotted by many small punctate basophilic-stained particles. In the periphery, remnants of cells which were undergoing necrosis were seen. External to this zone there were many large, swollen, histiocytic cells which were choked with small, round, basophilic particles. Large numbers of multinucleated giant cells were seen. These assumed very large proportions of bizarre appearance; many possessed from two to five nuclei which were either lobulated, indented, vesicular, or pyknotic. The cytoplasm was quite acidophilic. Abundant large mononuclear giant cells frequently showing granulation were also present. These elements represented foci of erythroporesis and myelopoiesis as well as the appearance of megakaryocytes.

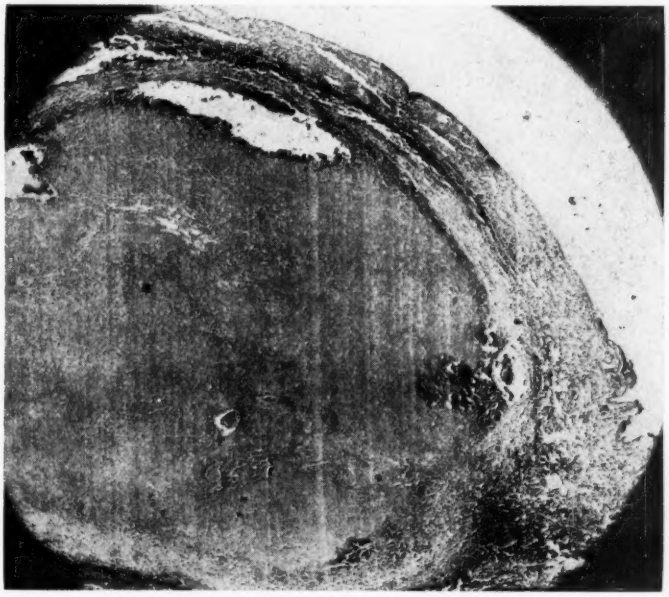

Fig. 6.—Photomicrograph of the submucous hematomas of the bladder (×16).

Lymph nodes from the radix mesenterii, para-aortic, and para-pancreatic region were studied. Qualitative alterations were quite similar in all nodes. There was considerable engorgement throughout, the capillaries being particularly distended. Foci of hemorrhage were frequent. There was a network composed of well-developed, fibrous septae which arose in the capsule or hilus and which created a lobular appearance. The usual nodal pattern was absent and lymphocytes were distributed rather evenly. Germinal centers were completely absent and there was no focal accumulation of lymphocytes. Amidst these cells were strewn innumerable large monocytoid cells possessing eccentric reniform or lobulated nuclei, the chromatin of which was usually reticulated or Rad-kern in appearance. These cells possessed definite cell boundaries; the cytoplasm was usually somewhat opaque and nongranular in appearance. Many had a strong acidophilic cytoplasm. A moderate number of large multinucleated and multilobulated cells were strewn irregularly throughout the tissue. Frequently the giant cells possessed mitotic figures. The peripheral sinuses were not particularly swollen. They contained a moderate number of round and reticulum cells, some of

which were swollen and contained a brown-yellow clumpy or granular pigment. Basophilic nuclear debris frequently appeared to have been engulfed by phagocytic cells.

The serosal surfaces of the gastrointestinal tract were coated by a thick mass of hemorhagic plastic exudate.

Kidney.—There was considerable diffuse, as well as patchy, congestion throughout the parenchyma, especially in the cortex. The glomeruli were considerably engorged but showed no degenerative changes. The tubular epithelium was swollen, granular, and vacuolar and had desquamated in some places, forming solid plugs. In several places dry, contracted, hemoglobin casts were noted. There were many foci of lymphocytic and plasma cell infiltration, especially in the perivascular tissues.

Bladder.—Sections through the tumor mass of the bladder wall disclosed the epithelium to be completely absent. The lamina propria was edematous and infiltrated with many large macrophagic cells containing brown pigment. Between the muscle coat and the mucosal layer, there was a large, firm clot showing some degree of organization (Fig. 6). All coats were diffusely hemorrhagic.

Bone Marrow (Direct Smear and Fixed Specimens).—Bone marrow consisted of broad sheets of cells with comparative absence of fat. There was considerable congestion of the sinusoids. The cells could be readily identified as members of the granulocytic series, including myeloblasts, a moderate number of myelocytes, and juvenile, stab, or segmented forms.

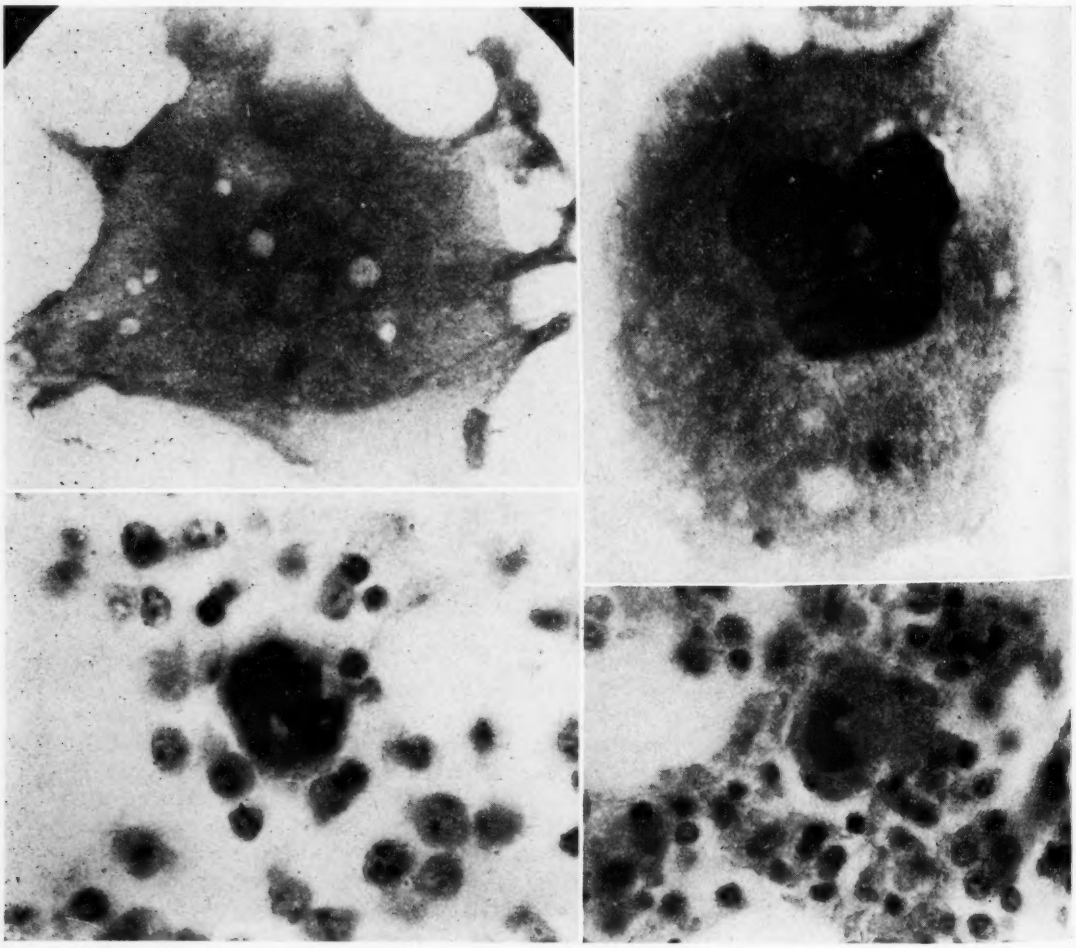

Fig. 7.—*A* and *B*, Photomicrographs of megakaryocytes on direct smear obtained by sternal puncture. These illustrate the severe toxic vacuolization of the cytoplasm (×1400). *C* and *D*, Photomicrographs of megakaryocytes from fixed bone marrow obtained at autopsy. Here the bizarre-shaped, pyknotic nuclei are clearly evident (×900).

There were, also, considerable numbers of normoblasts and nucleated red cells. Megakaryocytes were present in abundance. These showed considerable evidence of hyperplasia demonstrated by the large, swollen, nonlobulated, juvenile forms. Adult forms could be seen but these disclosed considerable degenerative alterations characterized by the presence of pyknotic nuclei, vacuolization of the cytoplasm, and loss of granulation (Fig. 7 *A*, *B*, *C*, *D*).

DISCUSSION

The diagnosis of thrombocytopenic purpura in this case appears to be well supported on the basis of consistently low platelet counts, absence of clot retraction, somewhat prolonged or normal bleeding time, normal coagulation time, the positive Rumpel-Leed phenomenon, positive venom skin test associated with spontaneous purpura, and free bleeding from the bladder mucosa. The anemia was not out of proportion to the amount of bleeding; there were no pathologic cells in either the blood or bone marrow diagnostic of leucemia or specific anemic states (aplastic anemia, pernicious anemia, chronic hypochromic anemia), and there was no appreciative enlargement of the spleen, further ruling out the group of congestive splenomegalies.

Because of the marked reduction in the number of blood platelets, consideration of nonthrombocytopenic hemorrhagic disorders may be safely omitted. A diagnosis of Schönlein's purpura is not valid, since the arthritic manifestations antedated the hemorrhagic phenomena by several months. Henoch's purpura may also be excluded, since the abdominal pain, epigastric distress, hematemesis, and melena diagnostic of this condition were consistently absent. Vitamin C or K deficiencies, hemophilia, hereditary fibrinogenopenia, multiple hemorrhagic telangiectasia, and hereditary hemorrhagic diathesis are not supported by any evidence on hand.

With the primary diagnosis thus assured, further analysis of the case is mandatory in order further to classify the syndrome as either essential, primary, or as secondary, symptomatic, thrombocytopenic purpura. Our patient was a Negro man, aged 35 years, a fact which poses several preliminary questions of probability. According to Wintrobe, Hanrahan, and Thomas,[13] and Limarzi,[8] essential thrombocytopenic purpura is exceedingly rare among the Negro race. Extremely striking is the high incidence of cases in young female adults. Wintrobe, Hanrahan, and Thomas found that 88.7 per cent of the cases occurred before the age of 24 years. No more than 10 per cent of the cases were over 40 years of age. In their series, Wiseman, Doan, and Wilson[14] found 60 per cent before 21 years and 50 per cent before adolescence.

On the basis of these statistical data, the chances of a male Negro, 35 years of age, suffering from essential thrombocytopenic purpura are reduced. Since the development of essential purpura hemorrhagica is much more common in childhood, the appearance of purpura in an adult should arouse suspicion of a chemical etiologic agent.

The evidence in favor of toxic, secondary thrombocytopenic purpura in our case is quite impressive. This will be presented from the standpoints of the history of exclusive medication with sodium salicylate, the failure of splenectomy to cure, and morphologic data.

The History of Exclusive Medication With Sodium Salicylate.—Sodium salicylate was started Jan. 31, 1944, and consisted of a mixture of unknown amount which was given every four hours for three days on the British hospital ship. After admission to a numbered general hospital, this therapy was continued, 80 gr. daily, from Feb. 4, to March 8, 1944, which totals 2,640 gr. in

thirty-three days. From March 8 to March 22 the patient was enroute from overseas to Fletcher General Hospital and no medication of any kind was given. The patient was started on sodium salicylate March 22, with a daily dose of 30 gr., and this medication was continued until and including April 1. A total of 300 gr. were given in this ten-day period. No other drug except an occasional multivitamin capsule was given the patient.

It is significant that the patient was exposed to a comparatively large amount of sodium salicylate (2,640 gr.) for thirty-three days without demonstrating any hemorrhagic phenomena. This was followed by a drug-free phase of fifteen days, at the end of which he was once again exposed to the same noxious agent. Nine days after onset of this second exposure, the bleeding began. This behavior is so similar to the course of events in "serum sickness," for instance, that it is difficult to conceive of a mechanism other than an antigen-antibody reaction of allergic nature. The nine-day period of latency following re-exposure in our case is classical for other allergic states.

A similar behavior has already been recorded in a number of cases of thrombocytopenic purpura due to Sedormid, a notorious offender.[5] Although large amounts of this drug had been taken for a prolonged period of time (several months) without untoward effects, injection of a comparatively small dose after a short period of abstinence was followed by purpura. The deliberate administration of small amounts of Sedormid and of organic arsenicals[4] caused the development of purpuric and ecchymotic lesions within a short time, and, in the case of arsenicals, varying degree of shock. Peripheral thrombocytopenia has been observed in experimental anaphylactoid shock,[7] although the degree of platelet reduction had been variable. Allergic sensitivity to vegetable foods, snake venoms, and insect bite has been incriminated in purpura. To recapitulate, it is probable that the patient became sensitized to the antigen (sodium salicylate) during his hospitalization overseas, which stimulated the production of sodium salicylate antibody in his serum. Upon readministration of the specific antigen, the allergic reaction occurred nine days after onset of exposure and expressed itself in the form of a hemorrhagic diathesis.

The Failure of Splenectomy to Cure.—The megakaryocytes in bone marrow examination were found in ample numbers. At the time of examination, the significance of their qualitative morphologic changes was not appreciated and, thus, on the basis of these alterations, no objection could be raised against splenectomy. Personal communications from Dr. Charles A. Doan, following the fait accompli, enlightened us as to the correct interpretation of these abnormalities and furnished additional evidence for the belief that this was a secondary thrombocytopenic purpura. In the light of his experience, vacuolated cytoplasm, pyknotic nuclei, and absence of granulation constitute evidence of a toxic effect. In addition to these changes, the presence of metaplastic foci of megakaryocytes in the spleen is further substantiation of the toxic genesis.

Morphologic Data.—Failure of splenectomy to cause cessation of the hemorrhages is introduced as valid evidence to support the diagnosis of secondary toxic thrombocytopenic purpura due to sodium salicylate, since it has been definitely established that this operation is particularly effective in controlling the hemorrhages of primary thrombocytopenic purpura.

Doan,[3] and Wiseman, Doan, and Wilson[14] stress the contraindications of splenectomy in cases of doubtful diagnosis, cases presenting a recent history of

contact with certain drugs, cases occurring during the actual or convalescent stages of all infectious diseases, and cases in which the bone marrow shows depletion of the megakaryocytic content associated with morphologic abnormalities. These mandates have secured universal acceptance. In our case, the absence of a history of previous medication with sodium salicylate, the lack of a precedent of the thrombocyte depressing action of this drug, and the unawareness of the significance of the morphologic changes in the megakaryocytes prevented the establishment of proof of an allergic reaction. In view of the fact that the prognosis was considered grave, and because 20 liters of blood had been given without improvement, splenectomy was performed as a last resort.

CONCLUSIONS

1. A case of fatal secondary toxic thrombocytopenic purpura due to sodium salicylate is presented.

2. The causal relationship is based on the proved history of sensitization to sodium salicylate, the absence of exposure to any other chemical or infectious agent, the morphologic characteristics of the megakaryocytes, the metaplastic appearance of these elements in the spleen, and the failure of splenectomy to improve a thrombocytopenic purpura.

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THE MORPHOLOGY OF THE ERYTHROGENIC RETICULUM OF THE BONE MARROW IN ADDISON-BIERMER'S DISEASE IN RELAPSE AND EARLY REMISSION AS SEEN BY THE IMPRINT METHOD

With Reference to the Myelogenic and Megakaryocytogenic Reticulum

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THE morphology of the bone marrow organ in Addison-Biermer's disease (addisonian pernicious anemia) has received considerable attention with the advent of intravital bone marrow studies for diagnostic, therapeutic, and prognostic purposes. A critical analysis of the pros and cons pertaining to the character of the erythro-, myelo-, and megakaryocytogenesis revealed that the structural pattern of the reticulum has been little studied. The reasons for the treatment of the reticulum as a minor subject are (a) the widespread presumption that the absence of the so-called "antipernicious anemia principle" (erythrocyte maturation factor) brings about a "maturation arrest" of the erythroblast (megaloblast), myeloblast, and megakaryoblast, (b) the use of the term "megaloblast" to designate the earliest recognizable erythroblast and the belief of Sabin[19] that this erythroid cell is morphologically identical with the basophilic "megaloblast" in the marrow affected by Addison-Biermer's disease, and (c) the widely accepted concept of Doan,[4-6] that the erythroid cell is derived from the endothelium of "intersinusoidal capillaries." These factors have led to diametrically opposed opinions as to the type of erythroblasts present in the normal marrow and in the marrow affected by Addison-Biermer's disease[9, 11] and as to the mechanism by which normal erythropoiesis is re-established.

In order that the reader may more fully appreciate the significance of the statement just made, a few of the most recent discourses pertaining to the type of erythrogenesis present in the marrow in relapse and early remission of Addison-Biermer's disease will be briefly discussed. Schartum-Hansen[20] believes that the promegaloblast is the precursor (primitive form) of the macroblast (pronormoblast of other authors). Under specific therapy the promegaloblast differentiates into a macroblast and the latter into a normoblast. According to Lambin and DeWeerdt,[14] the promegaloblast may either differentiate into an intermediate proerythroblast "erythroblastes intermediaires" which matures into a polychromatic megaloblast, and at this maturation stage the identity of the cell is lost, or the promegaloblast may develop directly into a pronormoblast and normoblast, respectively. Davidson and associates[1,3] refer to the promegaloblast as type I erythroblast, which, according to these investigators, includes the proerythroblast and primitive megaloblast of other authors. This type I cell, which is present in the normal bone marrow, is increased in addisonian pernicious anemia. Under specific therapy it develops into a type II (morphologically similar to the intermediate erythroblast of Lambin and De-

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Weerdt) and subsequently into a type III erythroblast, that is, the normoblast. Wilton[32] presumes that the development of a megaloblast is the sequela of an abnormally slow maturation of a cell he designates "reticulo-erythroblast or erythro-myeloblast," morphologically identical with the "granulomyeloblast" but biologically different. He believes that megaloblastosis is the expression of a disturbed tissue development. The alteration of the nuclear morphology (nuclear maturation arrest) would be proportional to the degree of the pathologic physiology. Wilton assumes that liver therapy accelerates the maturation process and restores normal erythrogenesis. Vischer[34] is of the opinion that the megaloblast is not transformed into a normoblast. This view is shared by Limarzi[16] and Rohr.[18] Koller[13] believes that a "mehrkerniger Megaloblast" or "Giantoblast" gives rise, by the process of heteroplastic division, to as many as three normoblasts. The latter cell type is further provided by small megaloblasts, "Übergangsformen," which develop into normoblasts, whereas other normoblasts are produced by homoplastic development of retained erythroid cells. Jones,[8-11] who derives the erythroblast from the myeloblast and reticulo-endothelium, believes that the vast majority of megaloblasts can be traced to the myeloblast. He states that the "megaloblast" is a pathologic cell and that the normoblasts which constitute part of the conversion pattern are derived from remnants of the normoblastic series and by heteroplastic development from the reticulum, the myeloblasts, or both. Limarzi and Levinson[15] hypothesize that the rapid transformation of the megaloblastic to normoblastic marrow is caused by multipolar mitosis of a stem cell (which, according to their hypothesis, originates from the reticulum) and by large multinucleated erythroid cells which give rise to normoblasts only. Doan[6a] is of the opinion that a maturation arrest of the megaloblast occurs because of lack of the erythrocyte maturation factor which, in turn, results in a nonutilization of iron considered essential for the maturation of erythroid cells. Administration of specific therapy results in the utilization of iron and production of early and late erythroblasts and normoblasts, respectively. Watson and associates[31] suggest on the basis of protoporphyrin studies that the reticulocytes appearing after liver therapy may be derived from megaloblasts.

I[24] have stated elsewhere my opinion (a) that the promegaloblast is a pathologic cell and is derived from a diseased reticulum and (b) that this early erythroblast does not give rise nor develop into a pronormoblast or basophilic normoblast and vice versa. Furthermore, I expressed the belief that the promegaloblast characterizes the marrow morphology of patients with Addison-Biermer's disease per se and biologic variations* of this hereditary and complex clinical entity. This concept is strengthened by the observations presented in this paper.

The preceding brief outline of the most recent hypotheses regarding the character of the physiologic and pathologic erythrogenesis and the process by which the latter state subsequently is corrected shows that the subject is of a highly controversial nature.

As to the morphology of the reticulum, Schulten[26, 27] observed that the "Retikulumzellen sind meist sehr zahlreich und gross." Vischer[34] remarks that the reticulum is "einem eigentümlichen Mark-Wucherungsprozess unterworfen." Liver therapy, according to him, brings about a retrogression of the reticulum hyperplasia. Rohr[18] speaks of a "stark proliferierenden Retikulum." Jones[11] suggests a probable involvement of the reticulo-endothelium.

*Group of diseases genetically related to Addison-Biermer's disease. Such a variable may be identified by the morphology of the reticulum and presence of promegaloblasts of the type described in this paper.

It is the purpose of this paper to show (a) that Addison-Biermer's disease (addisonian pernicious anemia) involves the reticulum, (b) that the reticulum is pathologically activated, (c) that the lack of the liver principle does not cause a maturation arrest of the normal erythroblast but that a pathologic erythroid cell the "promegaloblast" is formed, (d) that organotherapy (specific therapy) affects the reticulum, and finally (e) to re-emphasize the close relationship between the reticulum and the so-called stem cells, the erythroblast, myeloblast, and megakaryoblast, by showing that these cells retain structural patterns identical with the activated reticulum which proliferated them.

MATERIAL

One hundred two patients (53 men and 49 women) with uncomplicated Addison-Biermer's disease (addisonian pernicious anemia) in relapse served as subjects. These individuals were under the care of the medical service of the Minneapolis General Hospital. With the exception of four patients who showed free hydrochloric acid in the gastric juice after histamine stimulation,* all subjects presented clinical and hematologic features characteristic of Addison-Biermer's disease in relapse. The insidious onset of this hereditary and complex disease makes it practically hopeless to fix the date of the first symptoms, the number of attacks, and the spontaneous remissions preceding the progressive downhill course which forced the patient to seek medical attention. For practical and statistical purposes, these patients were therefore classified "first admissions," meaning that none of them was under the observation of a physician and had no specific therapy previous to hospitalization. The majority of the group was of Scandinavian stock. The age of the patients ranged between 29 and 88 years, with a mean of 59.3 years for the male and 65.8 years for the female. The mean age for the entire group was 62.5 years. The circulating erythrocyte quantity ranged between 480,000 and 2,800,000 cells per cubic millimeter. The mean corpuscular volume was between 98.6 and 160 cubic microns. The reticulocyte percentage was between 0.3 and 1.5 per cent. The sternal part of the bone marrow organ was hyperplastic in the majority of the patients. Promegaloblastosis was conspicuous in all marrow specimens. The sternal marrow was re-examined twenty-four, thirty-six, forty-eight, and seventy-two hours after specific therapy had been instituted. The distribution of therapy was as follows. Sixty patients received parenteral liver extract, 1 U. S. P. unit, and ten subjects, 15 U. S. P. units daily. Twenty patients were given Ventriculin,† 40 Gm., equal to 1 U. S. P. unit daily. Seven received the juice of one pound of raw liver daily. Five patients recovered from the anemic state without specific therapy (spontaneous remission). These patients served as controls. Another bone marrow aspiration was done on these control subjects at the peak of reticulocytosis to supplement the specimen obtained on admission.

NOMENCLATURE

The term "relapse" connotes the characteristic bone marrow morphology and peripheral erythrocyte quantity of less than 3.0 millions per cubic millimeter. The terms "early remission" and "late stage of early remission" are used

*I am aware of the accepted medical dictum "no anacidity, no addisonian pernicious anemia." I do not believe in such a restricted limitation as far as biologic processes are concerned. Modifications occur in other clinical entities; thus biologic variations should be expected in this disease also.

Over a period of from one to two and one-half years secretion of free hydrochloric acid ceased in all four subjects. The fact that achlorhydria developed and symptoms of relapse occurred when specific therapy was withdrawn shows clearly that the concept of "biologic variations" is correct.

†Parke, Davis & Co., Detroit, Mich.

for the healing phase, that is, adjustment of the pathologic state of the marrow organ toward normality under specific therapy or by means of spontaneous elaboration of the liver principle. The term "reticulum" is applied to quiescent totipotential mesenchymal cells (lymphoid reticulum) and activated reticulum. The terms "erythrogenic reticulum, myelogenic reticulum, and megakaryocytogenic reticulum" denote activated reticulum which, from the morphologic point of view, is distinctly associated with the proliferation of erythroblasts, myeloblasts, and megakaryoblasts. The term "erythroblast" is used in a collective sense and covers any nucleated erythroid cell in both the normal and pathologic marrow organ. The term "promegaloblast" as defined by Naegeli is applied to the earliest recognizable pathologic erythroblast. The term "pronormoblast" is applied to the earliest recognizable normal erythroblast. The term "myeloblast" as defined by Naegeli is used for the earliest recognizable myeloid cell, and the term "megakaryoblast" is applied to the stem cell associated with thrombocytopoiesis.

METHOD FOR OBTAINING GROSS MARROW UNITS FOR IMPRINT AND HISTOLOGIC PREPARATIONS

A detailed account of a method for obtaining gross marrow units from the human sternum has been published elsewhere.[23] Until the inexperienced investigator is familiar with the material from which he is to make "key" imprint and histologic preparations for the purpose of studying the morphology of the reticulum, the aspirated sternal marrow should be gently mixed with finely powdered commercial heparin.* Key imprints are made without the benefit of an anticoagulant from gross marrow units immediately when aspirated. The aspirated marrow is spread thinly over a paraffin-covered glass plate. A large gross marrow unit is picked up with the flat cut end of a wood applicator. Care must be taken that the tissue be only slightly touched and that it be ever so slightly spread for several millimeters on a chemically clean glass slide. This procedure is repeated until several rows of serial imprints of the marrow tissue have been made.[25] The opposite cut end of the wood applicator is used for picking up another gross marrow unit. This method requires some skill and speed because the gross marrow units must be picked up and the imprints made before a coagulum is formed. The method just described produces a minimum degree of cellular distortion; that is, delicate structural details are preserved. The use of an anticoagulant, however, permits not only transportation of the marrow specimen to the laboratory, but also the selection of gross marrow units suitable for imprint and histologic preparations to be done in a leisurely manner. While the photomicrographs which accompany this article were made from gross marrow units not exposed to an anticoagulant, it has been my experience that for as long as thirty minutes no appreciable changes occur in the architecture of the units and nuclear morphology if the marrow specimen has been kept fluid by heparin as recommended in this paper. It is suggested that the effect of new anticoagulants on the cellular morphology be compared with that of a key imprint. The imprint preparations are stained according to a method published elsewhere.[21, 25] Briefly the procedure is as follows:

1. Whip preparation through the air to facilitate drying.
2. Bring the slide in a horizontal position.
3. Place 0.5 c.c. of Wright stain† on the slide and permit the dye to act for two minutes.

*Commercial grade derived from dog liver (Lots 151 and 152) is recommended. Made by Hynson, Westcott and Dunning, Baltimore, Md.

†Only a certified stain should be used and made up according to directions on the label.

4. Add 2 c.c. of distilled water (pH 6.0 to 6.4). Place a paraffin-coated wooden applicator horizontally over the fluid surface (long axis of the slide) and keep the mixture in motion by removing and reapplying the applicator to the surface of the staining mixture. Repeat this step until stain and diluent are well mixed.

5. Permit the stain to act for from five to ten minutes.

6. Rinse the stain off with distilled water.

7. Dip the slide for several seconds in Schleicher's decolorizer.*

8. Wash well under running distilled water or two changes of distilled water.

9. Check staining under the microscope while preparation is still wet.

10. Let the preparation dry by air.

The gross marrow units selected for histologic preparations are fixed in a mixture consisting of physiologic saline, 90 c.c., and neutral formaldehyde, 40 per cent solution, 10 c.c. Helly's fixing fluid may be used. Fixation in the latter fluid should not exceed one hour. The units should be serially sectioned about 5 microns in thickness. The sections may be stained either with azure II and eosin, hematoxylin and eosin, or any other preferred routine staining method.[26]

GENERAL CONSIDERATIONS PERTAINING TO THE STUDY OF ACTIVATED RETICULUM

While the nature of the mechanism that initiates the development of a quiescent reticulum cell into an erythroblast, myeloblast, or megakaryoblast is still unknown, it appears from the study of activated reticulum that the stimulus is a "specific." When presumably the substance becomes structurally different, or is quantitatively, qualitatively, or both, deficient, the morphology and functional state of the reticulum undergoes conspicuous changes.

Morphologic variations of activated reticulum may be encountered in the physiologic bone marrow organ. The presence of some malformed reticulum should be expected in a structure as complex as that of the bone marrow organ. It is obvious, therefore, that the investigator should be well acquainted with the variations of the morphology of the normal reticulum and artifacts created by the method employed in order that he be able to segregate the aforementioned structural deviations from those caused by Addison-Biermer's disease.

It became apparent from the study of normal and pathologically activated reticulum by the imprint method that the erythrogenic, myelogenic, and megakaryocytogenic reticulum cells have distinct patterns which appeared to be sufficiently constant to permit an observer to distinguish between the various types of reticulum. The pathologically activated erythrogenic reticulum may be divided into three arbitrary patterns which, like all biologic phenomena, are subject to variations. Although the variability does not deviate to such a degree from the standard patterns described here that difficulties arise regarding a clear-cut separation of the patterns, the latter are (a) the morphology of the erythrogenic reticulum in relapse, (b) the morphology in early remission, and (c) the morphology in the late stage of early remission.

While a detailed description of the structural pattern of the erythrogenic reticulum is given in this paper, it should encourage the observer to "see" variations of morphology rather than to fix a too limited pattern in his mind because even the experienced morphologist at times may have trouble classifying activated reticulum according to a static pattern, particularly when the pathologic process is very severe.

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*Acetone 0.5 c.c.
Methyl alcohol 5.0 c.c.
Distilled water 100 c.c.

THE MORPHOLOGY OF THE ERYTHROGENIC RETICULUM IN RELAPSE

The activated erythrogenic reticulum in relapse presented in general the morphology shown in Fig. 1, *A*. Comparison with the normal erythrogenic reticulum, Fig. 4, *A* (assumed to be at the same developmental stage), reveals several conspicuous differences in morphology. The chief features are the greater size of the reticulum syncytia and particularly the pattern of the nuclei. Promegaloblasts are in the process of proliferation, are attached to, or are in the immediate proximity of the erythrogenic reticulum. The lower arrow in Fig. 1, *A*, points to a reticulum nucleus accumulating a cytosome. The nucleoli are not conspicuous. The latter structures are not easily demonstrable because of their presumed fluid state and variability of compressed chromatin about these complex structures. The nuclear chromatin has a fine granular appearance and is uniformly distributed throughout the well-defined parachromatin. The nucleus by its pronounced affinity for basic dyes is sharply outlined against the forming cytosome which ranges in hue from medium to light blue. A nuclear envelope (membrane) is not particularly discernible. It is believed that because of accelerated anabolic and catabolic metabolic processes the envelope is neutral and thus fails to stain distinctly. A perinuclear zone and Hof, whose purpose seems to be to permit a free exchange of metabolites between the cytosome and nucleus, and, more indirectly the nucleoli, appears at this developmental stage. The rate of cellular dehydration influences the amount of precipitation of colloid proteins and other constituents momentarily present within the zone and Hof, and thus granules may or may not be present within these areas.[22]

The constituents of the remaining cytosome are seemingly of a more viscous character than those of the zone and Hof. Thus the cytosome is more or less sharply demarcated from the common cytoplasm of the reticulum. This demarcation is particularly true at the periphery of the cytosome. No correlation by actual measurements could be made out between the severity of the anemic state and the size of the erythrogenic reticulum and nuclei. The tendency toward hugeness of both structures is, however, striking. In general, the erythrogenic reticulum stains more brilliantly and lighter than the normal reticulum and deeper than the myelogenic reticulum. From the morphologic viewpoint it appears that promegaloblasts (Fig. 1, *B*) are proliferated by the reticulum by means of the heteroplastic process because mitosis of this cell type has not been observed. However, mitosis is the mechanism by which the reticulum multiplies itself, and thus the presence of a mitotic figure indicates that the cell is most likely an activated reticulum cell rather than a promegaloblast. The morphology of detached promegaloblasts is shown in Fig. 1, *B*. The structural pattern corresponds well with those given in the literature.[9, 17, 33] Attention is directed to the nuclear and cytoplasmic morphology of the promegaloblasts as well as to their size, to the close similarity of morphology of those free cells, and to the cells which are in the process of forming but are still part of the reticulum. In this series the free promegaloblasts have been found to be larger than the pronormoblasts of the physiologic marrow. The cells classified as promegaloblasts range in size from 20 to 27 microns. These dimensions are far above those calculated for the pronormoblasts, which range between 13 and 16 microns.[22] Again no relation could be observed between the size of the free promegaloblasts and the anemic state. Thus it may be inferred that the genetic and constitutional background, at least to some extent, determine the type of cell proliferated.

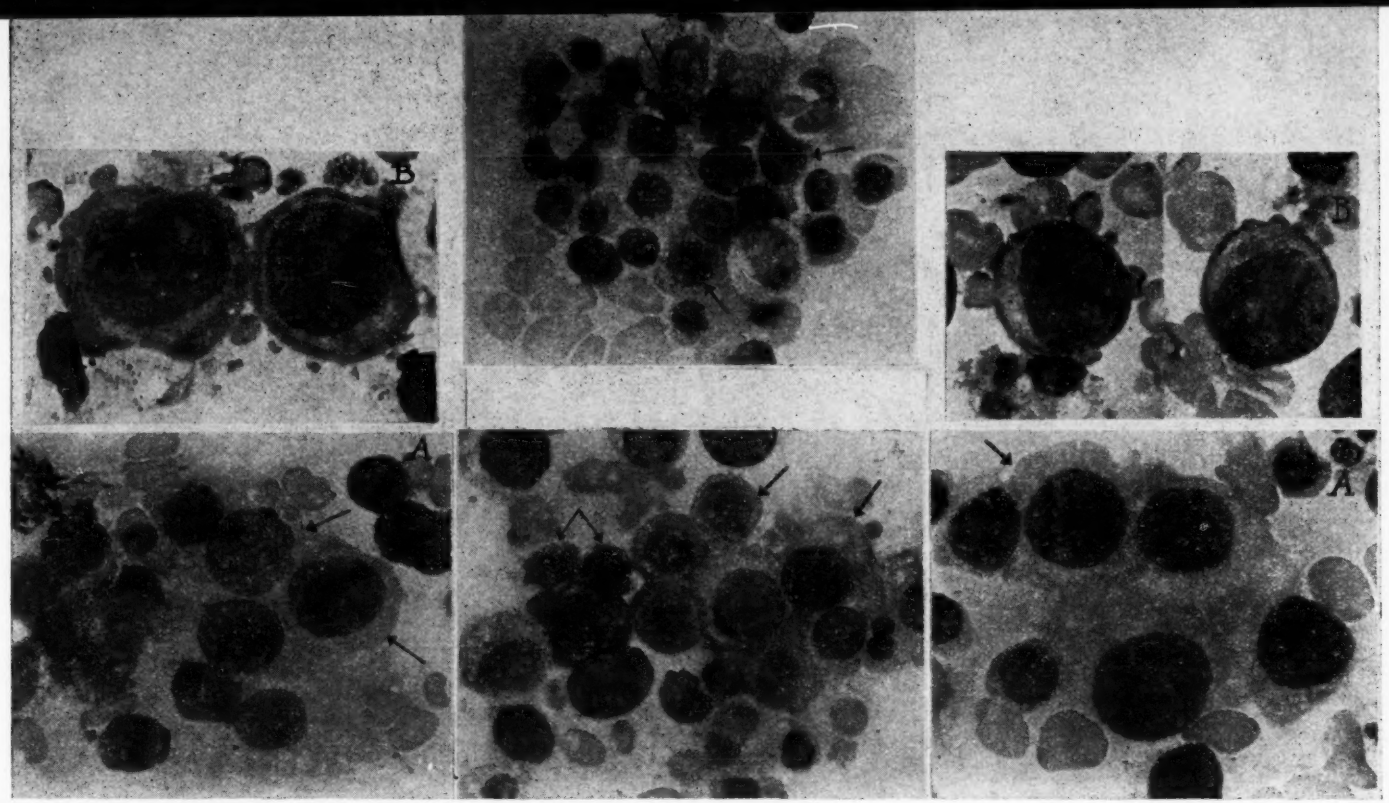

Fig. 1. Fig. 2 (Bottom) and Fig. 3 (Top). Fig. 4.

Fig. 1.—Addison-Biermer's disease (addisonian pernicious anemia) in relapse. *A*, Sternal marrow unit imprint showing pathologically activated erythrogenic reticulum. Upper arrow, reticulum nucleus of questionable vitality. Lower arrow, heteroplastic proliferation of a promegaloblast. *B*, Free promegaloblasts. Left, early promegaloblast, and right, late promegaloblast.

Fig. 2.—Addison-Biermer's disease (addisonian pernicious anemia), early remission, twenty-four to thirty-six hours after specific therapy. Sternal marrow unit imprint showing pathologically activated erythrogenic reticulum of the intermediate type. Twin arrow, extreme left, late promegaloblast, late telophase. The erythroblast is still attached to an early promegaloblast by a cytoplasmic bridge. Note the difference of the morphology of the nucleus of the "normoblast-like" megaloblasts. The extreme left erythroblast may be difficult to separate from a true normoblast of the same maturation stage. Arrows at the right show an early promegaloblast still attached to the common cytoplasm and a reticulum nucleus acumulating a cytosome.

Fig. 3.—Addison-Biermer's disease (addisonian pernicious anemia), late stage of early remission, forty-eight to seventy-two hours after specific therapy. Sternal marrow unit imprint showing slightly pathologically activated erythrogenic reticulum. Upper arrow, lymphoid reticulum cell. Arrow extreme right, physiologically activated reticulum cell. Lower arrow, slightly pathologic pronormoblast. Note the conspicuous adherence of the erythroblasts to the common cytoplasm.

Fig. 4.—Normal adult human sternal marrow. *A*, Sternal marrow unit imprint showing physiologically activated erythrogenic reticulum. Arrow points at reticulum nucleus accumulating a cytosome. *B*, Free pronormoblasts. Left, early pronormoblasts, and right, late pronormoblast.

Occasionally an erythrogenic reticulum fails to develop beyond the stage shown in Fig. 1, *A*. The common cytoplasm becomes a cytosome of several nuclei. Such a giant multinucleated erythroid mass may degenerate or break up into smaller masses. In a syncytium of the character just mentioned, one or all of the enclosed nuclei may undergo mitosis. The latter phenomenon is known as multipolar mitosis. This process may create the so-called "multinuclear giantoblasts" which, in turn, may or may not give rise to various types of giant erythroblasts.[7, 15, 24]

THE MORPHOLOGY OF THE ERYTHROGENIC RETICULUM IN EARLY REMISSION TWENTY-FOUR TO THIRTY-SIX HOUR PERIOD

Conspicuous changes occur in the morphology of the erythrogenic reticulum in the early remission phase induced either by specific therapy or by the mechanism of spontaneous elaboration of the so-called antipernicious anemic principle (erythrocyte maturation factor). In general, the reticulum is of a lesser magnitude—"intermediate reticulum." The size of the nuclei is correspondingly reduced; they are well demarcated but still show a fine granular chromatin. The parachromatin is prominent. The nucleoli still vary in character but are more sharply outlined. The nuclei are surrounded by a well-defined and more intensely blue-stained cytosome which is frequently connected by means of "cytoplasmic bridges" artifacts produced by vacuolation of the common cytoplasm. Promegaloblasts of lesser magnitude either are attached to or are in the immediate proximity of the erythrogenic reticulum. From the morphologic point of view, the cells correspond well to those described by Lambin and DeWeerdt as "erythroblastes intermediaires." Mitosis at that developmental stage is common, for heteroplastic cell production is at the minimum, and megaloblasts are now produced by the homoplastic process. At this particular stage the tendency to rupture of the reticulum syncytium and proliferated cells is conspicuously less.

In Fig. 2 is shown an example of an "intermediate reticulum." The top arrow points to a still attached promegaloblast. The extreme right arrow points toward a reticulum nucleus accumulating a cytosome. Two remnants of cytoplasmic bridges (pseudopods) are present. The twin arrow at the extreme left points at a late promegaloblast in mitosis. The erythroblast is still attached to an early promegaloblast. The difference in morphology of the nucleus of the "normoblast-like" megaloblasts is very striking. To classify correctly the erythroblast (at the left bar of the twin arrow) when detached from the erythrogenic reticulum is difficult indeed. Even an experienced morphologist may mistake such a cell for a true normoblast. This type of intermediate reticulum also characterized the marrow morphology of untreated individuals who recovered from the anemic state by spontaneous remission and of patients not included in this series whose erythrocyte quantities were above 3.0 million cells per cubic millimeter.

THE MORPHOLOGY OF THE ERYTHROGENIC RETICULUM IN THE LATE STAGE OF EARLY REMISSION FORTY-EIGHT TO SEVENTY-TWO HOUR PERIOD

In Fig. 3 is shown the phenomenal change of the morphology of the erythrogenic reticulum in the late stage of early remission. The dominant erythrogenic reticulum is characterized by a remarkable stability of the common cytoplasm and by a conspicuous coherence of erythroblasts of all maturation stages. The erythrogenic reticulum, however, bears the earmarks of having developed from

Fig. 4.—Normal adult human sternal marrow. *A*, Sternal marrow unit imprint showing physiologically activated erythrogenic reticulum. Arrow points at reticulum nucleus accumulating a cytosome. *B*, Free pronormoblasts. Left, early pronormoblasts, and right, late pronormoblast.

reticulum cells of various degrees of vitality. This assumption is believed supported by several conspicuous features: variability of the size of the erythrogenic reticulum, atypical mitosis, and, as the expected sequela, malformed erythroblasts. For example, the malformed pronormoblast in Fig. 3, bottom arrow, is easily distinguishable from the intermediate promegaloblast (Fig. 2, top arrow), although difficulties arise in predicting its developmental potentialities. From a purely hypothetical point of view, this slightly pathologic pronormoblast might give rise to normoblasts. The query arises as to whether this type of erythroblast can develop into an orthochromatic megaloblast in case the effectiveness of specific therapy should be modified in one way or another. The potential presence of pathologically activated erythrogenic reticulum may well account for the recurrence of megaloblasts. In Fig. 3 is further shown one physiologically activated reticulum cell (middle arrow), its nucleus being morphologically identical with the nuclei of the normal erythrogenic reticulum shown in Fig. 4, *A*, top arrow. Also shown in Fig. 3 is a presumed quiescent (inactivated) lymphoid reticulum cell (top arrow). Physiologic activation of such a reserve cell presumably may occur as early as twenty-four hours (and probably as late as five days) following the administration or spontaneous elaboration of the liver principle. The development of normal erythrogenic reticulum (Fig. 4, *A*) and pronormoblasts (Fig. 4, *B*) seems to be dependent upon two factors: (1) the number of quiescent lymphoid reticulum cells which become physiologically activated and (2) the reversibility to normal of only slightly pathologically activated reticulum cells. It is thus obvious that the momentary physicochemical state of the reticulum is of the utmost importance in organotherapy and recovery of the bone marrow as a whole.

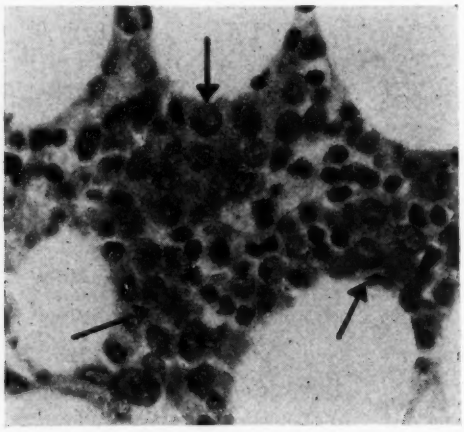

Fig. 5.—Addison-Biermer's disease (addisonian pernicious anemia), late stage of early remission. Histologic preparation of a sternal marrow unit. Stained with hematoxylin and eosin. Top arrow, pathologically activated erythrogenic reticulum. At the periphery, reticulum cells are undergoing mitosis. Lower left arrow, intermediate erythrogenic reticulum. Lower right arrow, physiologically activated erythrogenic reticulum. Note the normoblasts at the periphery.

In Fig. 5 is shown part of a serial section of a gross marrow unit obtained forty-eight hours after the administration of specific therapy. The top arrow points to an erythrogenic reticulum showing the characteristic relapse pattern. The three cells in the process of mitosis at the periphery are believed to be reticulum cells rather than promegaloblasts. The lower left arrow points toward an intermediate erythrogenic reticulum and the lower right arrow identifies a small and assumed normal erythrogenic reticulum to which are attached pronormoblasts and normoblasts.

The arbitrary division of the erythrogenic reticulum into three functional states according to morphologic patterns seems to furnish a legitimate basis upon which the effect of Addison-Biermer's disease on the reticulum of the bone marrow organ and, by the same token, the effect of specific therapy may be studied. It is believed that ample morphologic evidence has been presented to propose that Addison-Biermer's disease affects the reticulum and that the promegaloblast arises from a pathologically activated erythrogenic reticulum. Administration of the so-called antipernicious anemia principle does not only promote the development of the promegaloblast to a megalocyte (macrocyte of other authors), but also acts specifically upon the reticulum. According to this concept, pronormoblasts (Fig. 4, *B*) can be proliferated only by an erythrogenic reticulum that is in every respect physiologic.

THE MORPHOLOGY OF THE MYELOGENIC RETICULUM IN RELAPSE

The profound effect of Addison-Biermer's disease on the myeloid cells has been thoroughly studied by Tempka and Braun.[29] These investigators observed in the "Vollstadium" (relapse) various degrees of morphologic alterations of the myeloblasts. Analysis of the descriptions given of the morphology of the myeloblasts revealed that activated and free reticulum cells were included in this group of cells. Classification of free stem cells is dependent upon the knowledge of the morphology of the developing myelogenic reticulum. No reference is made by Tempka and Braun to the state of the reticulum. Jones[12] and Sharp and associates[28] noted pathologic alterations in the myeloid cells, but these investigators have omitted a detailed account of the structural pattern of the myelogenic reticulum.

In this series the activated myelogenic reticulum has been found to be easily distinguishable from the erythrogenic reticulum because of its less intense affinity for basic dyes, its morphologic pattern, and its larger size. In Fig. 6, *A*, is shown a characteristic pathologically activated myelogenic reticulum. Enclosed in this syncytium are several inactivated reticulum cells. The nuclei and cytoplasm of the assumed quiescent lymphoid reticulum cells have a striking affinity for basic dyes. The round to oval reticular nuclei contain from one to several inconspicuous nucleoli. The narrow, not well-defined cytosome contains from few to many small red granules and occasionally a few vacuoles. The cytoplasm varies in color from light to deep blue. The lower left arrow points to an inactivated reticulum and the right and upper right twin arrows point to various developmental stages of activated reticulum cells. The nuclei of the activated myelogenic reticulum may reach the size of the erythrogenic reticulum. The chromatin is in the form of fine strands (sievelike), in contrast to the granular form characteristic of the nuclei of the erythrogenic reticulum. The nucleus may show from one to several light blue nucleoli. The latter are sharply outlined by compressed chromatin during the differentiation process toward the myeloblast stage. The cytoplasmic mass is less bluish than the common cytoplasm of the erythrogenic reticulum at the same developmental stage. The individual cytosome is, therefore, not very discernible. The type of myelogenic reticulum described seems to proliferate pathologic myeloblasts. An example of the latter cell type is shown in Fig. 6, *B*.

In Fig. 6, *C* is shown an intermediate myelogenic reticulum which characterized the late stage of early remission of Addison-Biermer's disease. While this type of activated myeloid reticulum is conspicuously reduced in size and apparently is also functionally improved, it still proliferates more or less

pathologic myeloblasts. For comparative purposes, a representative of the normal type of myeloblasts proliferated by remnants of, or newly physiologically activated, myelogenic reticulum is shown in Fig. 6, *D*. It is as yet little understood why the return to normal myelogenesis lags so conspicuously behind the readjustment of erythrogenesis and, again, why it recovers more slowly than megakaryocytogenesis after specific organotherapy has been instituted. This phenomenon has been observed by various investigators, but no feasible explanation has been made. The reason for this sequence of recovery may well rest upon the order of development of the three systems in fetal life. The delayed recovery of the myelogenic reticulum may aid in diagnosing the disease when specific therapy has obliterated the pathologic erythrogenic reticulum.

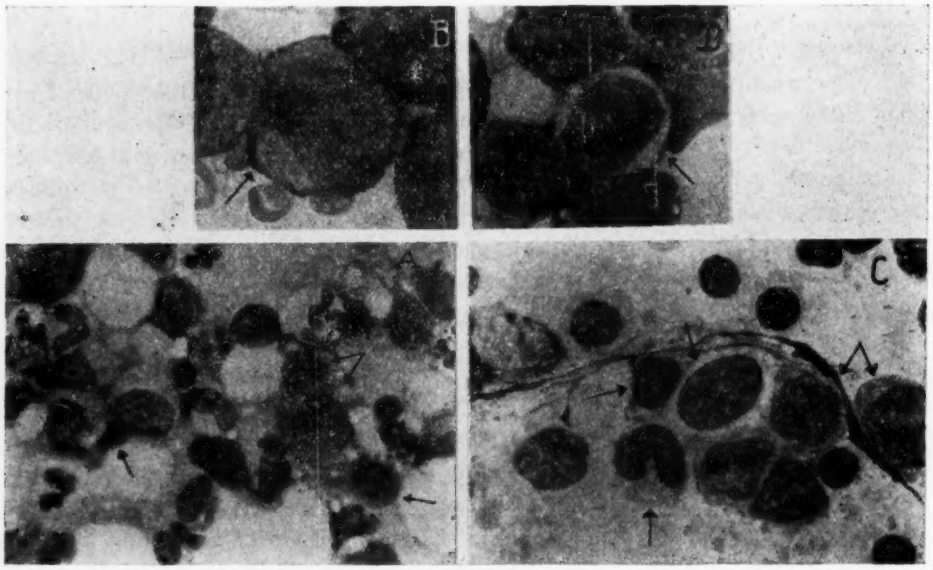

Fig. 6.—Addison-Biermer's disease (addisonian pernicious anemia) in relapse. *A*, Sternal marrow unit imprint showing pathologically activated myelogenic reticulum. Lower left arrow points to an inactivated and arrows at the right point at activated reticulum cells. *B*, Arrow identifies a pathologic myeloblast. *C*, Late stage of early remission intermediate myelogenic reticulum. Top arrow, slightly pathologic reticulum nucleus. Twin arrow, extreme right, shows an early and late myeloblast. Lower arrow points at a giant metamyelocyte. Arrow at extreme left, a lymphoid reticulum cell. *D*, At arrow, normal myeloblast.

THE MORPHOLOGY OF THE MEGAKARYOCYTOGENIC RETICULUM IN RELAPSE

Tempka and Braun[29] and Jones[12] have observed various degrees of morphologic alterations in the megakaryoblast, promegakaryocyte, and megakaryocyte, respectively, although the status of the megakaryocytogenic reticulum is not described. It must be pointed out that recognition of the megakaryocytogenic reticulum is difficult because it is very small compared to the erythrogenic and myelogenic reticulum. It consisted in this series of from one to two nuclei. Further, it appeared that the megakaryoblast becomes very easily detached from the common cytoplasm. It may be said that in general the nuclei of the megakaryocytogenic reticulum stain more intensely than those of the myelogenic reticulum. The nuclei are also larger and the chromatin is more coarse and forms irregular masses. The parachromatin is distinct and light red. Nucleoli are difficult to make out because they are generally covered over by chromatin, are small in size, and have a deep blue hue. A nuclear membrane is not discernible. The common cytosome is light blue and appears flecky. The nucleus accumulates a narrow cytosome when developing toward a megakaryoblast. The

cytosome of the latter is light blue and may contain a few reddish granules. Synchronized with the formation of a cytosome, an area develops in the middle of the nucleus which shows affinity for either acid or basic dyes. This vacuole-like structure varies in size in individual megakaryoblasts. The central location of the structure suggests a metabolic unit and gives the nucleus a doughnut appearance. In Fig. 7, *A*, the extreme left arrow points toward an activated reticulum nucleus. Its cytosome is not well defined. The right arrow identifies a megakaryoblast with a doughnutlike nucleus. The cytosome is narrow and still attached to the common cytoplasmic mass. The development of a central area is not a unique phenomenon of the pathologic megakaryoblast because a morphologically identical structure develops in normal megakaryoblasts also. An example of the latter is shown in Fig. 7, *B*.

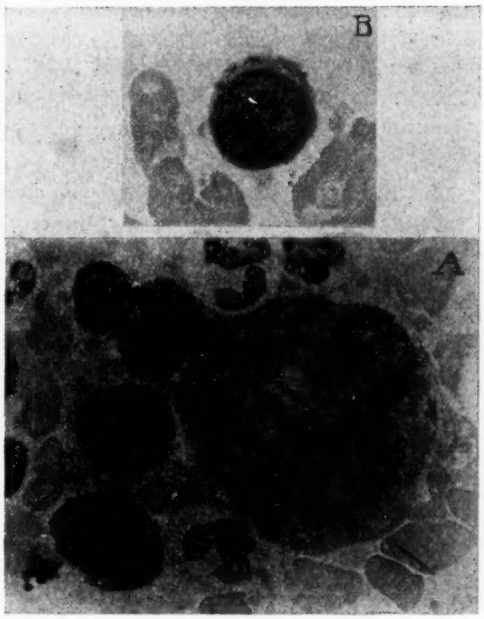

Fig. 7.—Addison-Biermer's disease (addisonian pernicious anemia) in relapse. *A*, Sternal marrow unit imprint showing a pathologically activated megakaryocytogenic reticulum. At arrow, extreme left, an activated reticulum cell. At the right arrow a pathologic megakaryoblast. *B*, Megakaryoblast from normal adult human sternal marrow.

DISCUSSION

It is the opinion of the majority of clinical investigators that Addison-Biermer's disease (addisonian pernicious anemia) is a deficiency disease. It is presumed that there is an absence of the intrinsic factor of Castle, although others believe that (a) the gastric principle is not absent but deficient qualitatively or quantitatively, or both, (b) that the intrinsic factor may be present in normal amounts but lack of an extrinsic substance causes a defective production of the gastric factor, and finally (c) that the gastric factor may not be absorbed by the intestines. Whichever theory will become an actuality, the fact remains that the reticulum is affected by the pathologic physiology underlying this hereditary and complex clinical entity.

In my collection of over 7,000 bone marrow preparations obtained during life and covering a large range of hemopathologic states, I have not observed morphologic alterations of the reticulum of the character described in this paper in diseases other than Addison-Biermer's disease and its biologic variables. On

the basis of the 495 marrow specimens obtained for this study, I favor the hypotheses (a) that the administration of specific therapy promotes the maturation process of the promegaloblast, (b) that the proliferation of the promegaloblast is inhibited by the elimination of the mechanism responsible for the pathologic activation of reticulum, (c) that specific therapy eliminates the promegaloblast by improving the metabolism of slightly diseased reticulum, and finally (d) that quiescent reticulum cells again are physiologically activated. It is presumed that these supposed actions of the liver principle are demonstrated by the changes in morphology of the erythro-, myelo-, and megakaryocytogenic reticulum cells. Nevertheless, the fact cannot be overlooked that any rationalization based upon morphologic patterns may suggest, but not necessarily represent, an actual biologic truth. The observation of Watson and associates that protoporphyrin values in Addison-Biermer's disease prior to treatment are low, increase slowly following liver therapy, and reach the highest level some time after the reticulocyte peak, and the presumption of these investigators "that the first mass of reticulocytes appearing after liver therapy may be derived from megaloblasts and may not contain as much porphyrin as those derived from normoblasts" appears to verify the morphologic data presented here. And again the type of erythroblast present in the early and late stage of early remission where the greater number of erythroblasts is made up of "normoblast-like" megaloblasts and of more or less malformed erythroblasts rather than physiologic normoblasts supports the deductions of Watson and associates. Physiologic erythroblasts when present were not prominent in the relapse and early remission phase in this series of cases. Thus it may be stated that the morphology of the reticulum reflects altered physicochemical states. Thus the hypothesis may be advanced that a "functionally altered reticulum is the sequela of a disturbance between the bone marrow reticulum and the substances and organs controlling its physiologic state." The injury to the reticulum by the disease process and the reversibility of the reticulum lesion may be adduced by means of morphologic evidence, although the final evaluation of the damage must await recovery of the bone marrow organ from the effects of the disease.

It is noteworthy that not all of the reticulum becomes diseased. It was observed that quiescent and apparently healthy reticulum cells were situated next to pathologically activated ones. This unaffected reticulum is apparently the reserve tissue upon which, in final analysis, the bone marrow organ reconstructs itself. Thus it may be presumed that, if a certain quantity of reticulum is destroyed by the disease process, the most likely sequela is a permanent hypoplastic state of the bone marrow organ. Furthermore, successive attacks and various types of senile changes within the organ will be a deciding factor regarding revitalization of the organ to a state where the cellular output meets physiologic values. It is believed that this statement is supported by the observation that, in spite of adequate and various therapeutic measures and absence of complications known to interfere with the recovery of the marrow organ, the peripheral erythrocyte quantities may remain between 3.0 and 4.0 millions per cubic millimeter. As a matter of fact, eleven subjects in this series have leveled out at and between these erythrocyte quantities. There is reason to believe that these erythrocyte levels represent the normal productivity of the respective bone marrow organ. In other words, re-establishment of physiologic hematopoiesis is dependent upon (a) the constitution of the bone marrow organ, (b) the degree of injury to the reticulum by a single or repeated attack of the disease, (c) the ability of slightly pathologically activated and quiescent healthy

reticulum cells to respond to the instituted organotherapy or to the mechanism of spontaneous remission. The study of the reticulum of the bone marrow organ affected by Addison-Biermer's disease has suggested that the common denominator for the characteristic erythro-, myelo-, and megakaryocytogenesis is not the expression of a maturation arrest of normal stem cells but is the product of a diseased reticulum.

The view presented in this paper concerning the attack of Addison-Biermer's disease on the bone marrow reticulum supports the statement of Rohr[18] that "die Verhältnisse wesentlich komplizierter liegen als bisher allgemein angenommen wurde" and the opinion of Naegeli[17] that "der Perniciosa eine ganz charakteristische, einheitliche und scharf ausgeprägte Funktionsstörung des Knochenmarkes zugrunde liegt."

SUMMARY

1. The morphology of the activated erythrogenic reticulum cell with reference to the myelogenic and megakaryocytogenic reticulum cells of the sternal part of the bone marrow organ in uncomplicated Addison-Biermer's disease (addisonian pernicious anemia) in relapse and early remission is presented.

2. One hundred two patients served as subjects. Four hundred ninety-five bone marrow specimens were obtained by means of the sternal aspiration method. Imprints of the gross marrow units were made and stained according to procedures described in this paper.

3. Sternal marrow was obtained before and after twenty-four, thirty-six, forty-eight, and seventy-two hours following the administration of specific therapy. Those patients who showed an intermediate reticulum pattern on admission were used for control purposes, and sternal marrow was obtained at the peak of the reticulocytosis only.

4. Morphologic evidence is presented which appears to favor the hypothesis that the disease affects the reticulum and that the latter is pathologically activated.

5. The morphology of the diseased reticulum in relapse and early remission and of the normal reticulum is sufficiently constant to permit an arbitrary division of the structural pattern of the erythrogenic as well as the myelogenic and megakaryocytogenic reticulum for the purpose of orientation regarding the effect of the disease and specific therapy on the reticulum.

6. The study of the reticulum suggested that re-establishment of normal hematopoiesis is dependent upon the reversibility of the reticulum lesion, the degree of physiologic activation, and the reproducing capacity of the newly activated reticulum.

7. The common denominator for the pathologic erythro-, myelo-, and megakaryocytogenesis which characterizes the marrow morphology in Addison-Biermer's disease (addisonian pernicious anemia) in relapse is a diseased reticulum.

8. Under specific therapy or spontaneous remission the diseased reticulum seems to become depleted and thus proliferation of pathologic stem cells ceases. The maturation process of the promegaloblast is apparently promoted, however, developing into a "normoblast-like" erythroblast rather than true normoblast.

9. The close relationship between the reticulum and the so-called stem cells, that is, the erythroblast, myeloblast, and megakaryoblast, is reemphasized by showing that these cells retain structural patterns morphologically identical with those of the respective activated reticulum.

10. It is pointed out that in a large collection of bone marrow specimens obtained during life covering an extensive range of hemopathologic states, morphologically altered reticulum of the character described in this paper has not been observed in diseases other than Addison-Biermer's disease. It is suggested that biologic variations of this hereditary and complex disease may occur. These genetically related clinical entities may be readily recognized by the morphology of the erythrogenic and/or the myelogenic and megakaryocytogenic reticulum as seen by the imprint method.

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THE CHOLERETIC ACTION OF THE ALLYL ESTER OF CINCHOPHEN (ATOQUINOL)

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CINCHOPHEN has been demonstrated to have choleretic properties in man and dogs but probably not in rabbits. This information has been available for some time but attention has been directed to it in recent years, especially by the work of Ivy and his associates, of Northwestern University,[1-3] who have reviewed the earlier literature pertaining to the subject. In the work to be described, it was found that in unanesthetized dogs with chronic biliary fistula, the allyl ester of phenylcinchoninic acid, commonly known under the trade name of Atoquinol, has choleretic properties which are qualitatively similar to those of cinchophen and quantitatively slightly different from them. Atoquinol was introduced into medical practice shortly after World War I under the name of Preparation 155G, or Atochinol.[9] There have been many favorable reports upon its therapeutic value in the treatment of gout and arthritic conditions.[5, 11] Gerbergas of Kaunas, Lithuania, working at the University of Geneva,[6] described atoquinol as "un médicament d'une très grande importance."

The technique used for collecting hepatic bile was analagous to that of Berman and co-workers,[3] with fifteen dogs, weighing between 10 and 20 kilograms, prepared after the manner described by Rous and McMaster.[10] The animals were given a full diet, rich in vitamins, and were exercised daily in fresh air about the lawns of the university campus. Hepatic bile was removed, measured, and analyzed daily. Atoquinol and cinchophen were given in a dose of 0.1 Gm. per kilogram of body weight orally as tablets impregnated into pieces of meat which the dogs readily swallowed. Because of the tendency in recent years to assume that drugs either augment the output of hepatic bile of normal composition (choleretics) or simply increase the water content (hydrocholeretics), it was decided to study as many properties of hepatic bile as possible. These studies are described herewith. It was found that both atoquinol and cinchophen, while augmenting the volume output of hepatic bile, had little effect upon the concentration of most ingredients investigated, but in a few instances concentrations or other properties were either increased

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or decreased. These results indicate that a simple classification into choleretics and hydrocholeretics does not tell the entire story.

The properties of dog hepatic bile before the administration of atoquinol or cinchophen, which might be termed normal or control hepatic bile, have been summarized in Table I. Following the administration of atoquinol or cinchophen, six of these properties were altered, the change usually occurring on the first, sometimes the second, day after giving the drug, and by the third day the alterations had returned to, or were returning toward, normal or control levels. In the instance of all properties, there was a change in the mean value after administration of the drugs, but in only six instances were these changes statistically significant. In some cases, the percentage change in the mean was not marked but was statistically significant, while in other cases the percentage change in the mean was considerably greater but the change was not statistically significant. It was therefore decided not to include the mean changes after administration of the drugs listed in Table I but rather simply to indicate if there was a significant change and, if so, what, and to describe the changes quantitatively in the text. The value of tables, charts, diagrams, etc., is to give the reader a quick, *correct* picture of what was found experimentally, and hence it is inadvisable to include changes in the mean which are not significant, as this gives an erroneous impression which has to be corrected by laboriously reading through the text.

TABLE I.—OUTPUT, PROPERTIES, AND COMPOSITION OF NORMAL, HEPATIC BILE COLLECTED FROM HEALTHY, UNANESTHETIZED, DOGS WITH CHRONIC BILIARY FISTULA AND EFFECT OF ORAL ADMINISTRATION OF 0.1 GR. PER KILOGRAM OF BODY WEIGHT OF ATOQUINOL AND CINCHOPHEN

ESTIMATION	UNIT	NORMAL VALUE (MEAN ± S. D.)	EFFECT OF ATOQUINOL AND CINCHOPHEN
Twenty-four hour volume	Ml. per kilo	7.4 ± 2.8	Significantly increased
Specific gravity	Gm. per ml.	1.0056 ± 0.0030	Significantly increased
Relative viscosity	Distilled H_2O=1	1.322 ± 0.337	Significantly decreased
Total solids	Gm. per 100 ml.	3.093 ± 0.124	Significantly increased
Sodium	Mg. per 100 ml.	350 ± 93	No significant change
Chloride	Mg. per 100 ml.	436 ± 113	No significant change
Potassium	Mg. per 100 ml.	34.8 ± 7.0	Significantly increased
Inorganic phosphate	Mg. per 100 ml.	20.6 ± 11.9	Significantly increased
Total fatty acids	Mg. per 100 ml.	317 ± 223	No significant change
Total cholesterol	Mg. per 100 ml.	52 ± 27	No significant change
Ester cholesterol	Mg. per 100 ml.	33 ± 25	No significant change
Free cholesterol	Mg. per 100 ml.	19 ± 10	No significant change
Bilirubin	Mg. per 100 ml.	38 ± 30	No significant change
Bile acids	Gm. per 100 ml.	2.54 ± 0.78	No significant change

The *twenty-four hour output* of hepatic bile before administering the drugs averaged 7.4 c.c. per kilogram body weight, a value lower than that occurring in man but comparable to that reported in dogs by other investigators. In over 90 per cent of experiments, the administration of atoquinol augmented the volume output of bile, the increase during the first day after the drug averaging 84 per cent, the second day 58 per cent and the third day 8 per cent. In all experiments, the giving of cinchophen increased the volume output of hepatic bile, the corresponding figures for the three days being 76, 133, and 27 per cent.

The *specific gravity* of bile was increased in approximately 95 per cent of experiments following the ingestion of atoquinol and in approximately 80 per cent following cinchophen. The actual percentage increases in specific gravity were, of necessity, small. The first day after atoquinol, the mean percentage

increase was 0.26 per cent, the second day 0.40 per cent, and the third day 0.15 per cent. The corresponding figures following cinchophen were 0.09, 0.46, and 0.25 per cent, respectively.

The *relative viscosity,* as measured with the Ostwald viscosity pipette, was lowered in from 85 to 90 per cent of experiments on the first, second, and third days following the administration of atoquinol, the mean percentage decreases being 6.6, 7.4, and 5.2 per cent, respectively. The relative viscosity was not significantly affected the first day after giving cinchophen, but on the second and third days it was lowered in 75 per cent of experiments, the mean decrease being small, 2.9 and 0.4 per cent, respectively.

Total solids were measured by drying aliquots of hepatic bile at 90° C. for 100 hours. The first day after giving the drugs, there was a significant increase in the total solids, an increase which occurred in some 75 per cent of experiments and which averaged 13 per cent following atoquinol and 7.8 per cent following cinchophen. On the second and third days, changes in the value for total solids were not significant following either of the two drugs.

Of the various solids measured, statistically significant changes were found only in the values for potassium and inorganic phosphate, both of which were increased, suggesting that atoquinol and cinchophen have an especial tendency to augment the output of potassium phosphate in bile. On the other hand, there was no significant decrease in the output or concentration of any of the other solids determined, to be described later, so that since the twenty-four hour volume was increased, there was actually an increase in the twenty-four hour output of all of these solids.

The *sodium content* of hepatic bile was determined after the method of Hoffman and Osgood.[8] The control value was 350 mg. per 100 c.c. of bile, and neither the administration of atoquinol nor of cinchophen significantly affected the mean concentration of hepatic bile sodium.

The *chloride content* of dog hepatic bile could not be shown to be affected in a statistically significant manner by the administration of atoquinol or cinchophen, although the mean values were slightly increased. Chloride was estimated by the method of Van Slyke[13] and the control concentration was 436 mg. per 100 c.c., with a standard deviation of 113.

The *potassium content* was determined after the method of Hoffman,[7] and a statistically significant increase could be shown to occur after the use of both atoquinol and cinchophen, the increase being more marked following cinchophen. After atoquinol, the mean percentage increase in hepatic bile potassium was 6.3 per cent on the first day, 10.9 on the second day and 11.8 on the third day. The corresponding values after cinchophen were, respectively, 17.7, 33.1, and 1.3 per cent.

Inorganic phosphates were estimated by an adaptation of the familiar Bell-Doisy technique to measurement in a photelometer. Both atoquinol and cinchophen produced statistically significant increases in the concentration of hepatic bile inorganic phosphate on the first and second days after administration but not on the third day. Following atoquinol, the mean percentage increase on the first day was 12.6 per cent and on the second day 6.8 per cent; following cinchophen, the percentage changes were considerably greater and averaged 44.3 per cent the first day and 40.9 per cent the second day. An increase in inorganic phosphates was found in between 70 and 80 per cent of experiments following atoquinol and between 80 and 90 per cent following cinchophen.

The *total fatty acid content* of dog hepatic bile was not affected in a statistically significant manner by the administration of either atoquinol or cinchophen. Total fatty acids were estimated after the oxidative method as modified by Boyd.[4] The normal mean was 317 mg. per 100 c.c., with considerable fluctuation in the individual values which was reflected in the high standard deviation as seen in Table I. On the average, atoquinol caused a slight decline in the concentration of total fatty acids and cinchophen a slight increase, but the changes were not marked nor constant and were statistically insignificant.

Total cholesterol, ester cholesterol, and *free cholesterol* were determined by oxidative micromethods as described by Boyd.[4] The values for normal dog hepatic bile, listed in Table I, are lower than those reported by investigators using colorimetric methods for cholesterol because, as is well known, cholesterol estimated in body fluids or tissues by the oxidative technique yields values which average from one-third to one-half lower than those recorded by the colorimetric methods. The administration of atoquinol or cinchophen had no consistent or significant effect upon the concentration of hepatic bile cholesterol.

Bilirubin was measured after the method of Thannhauser and Anderson.[12] Normal hepatic bile contained an average of 38 mg. per 100 c.c. of bilirubin and this mean was not significantly affected by the administration of atoquinol or cinchophen.

The *bile acid* content, measured with the photelometer and a quantitative test based upon the familiar Mylius-Pettenkofer reaction, had a normal average value of 2.54 Gm. per 100 c.c. and a standard deviation of 0.78. Neither atoquinol nor cinchophen significantly affected the bile acid content of dog hepatic bile.

SUMMARY

Atoquinol and cinchophen were given by mouth in a dose of 0.1 Gm. per kilogram of body weight to fifteen unanesthetized dogs with chronic biliary fistula, hepatic bile being collected and analyzed daily.

Atoquinol and cinchophen had a choleretic effect which was present for two days and was declining upon the third day.

There was little quantitive difference between the effects of the two drugs. Both increased the twenty-four hour volume output of hepatic bile, increased its specific gravity slightly but consistently and caused a decrease in its relative viscosity, an increase in the concentration of its total solids, an increase in its potassium content, an increase in its inorganic phosphate content, and no change in the concentration of sodium, chloride, total fatty acids, total cholesterol, ester cholesterol, free cholesterol, bilirubin, and bile acids.

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ANTICONVULSANT EFFECTS OF STEROIDS

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IT IS known that some steroids are able to influence cellular permeability as demonstrated by the observation of Tipton[1] that cortin depresses the release of potassium from stimulated muscles. Since cellular permeability in the central nervous system is an important factor in determining convulsive reactivity (Spiegel and Spiegel-Adolf[2]), it seemed of interest to study whether some sterols may have anticonvulsant properties. Such an expectation was supported by Selye's[3] observations of hypnotic effects. Preliminary experiments of Spiegel[4] demonstrated that desoxycorticosterone, testosterone, and progesterone are able to raise the threshold for electrically induced convulsions, and experiences of McQuarrie, Anderson, and Ziegler[5] with desoxycorticosterone on two patients with epilepsy seemed to point to a similar direction. In Spiegel's[4] experiments a margin between anticonvulsant and hypnotic dose could hardly be established. It seemed, therefore, desirable to extend these studies to a larger group of steroids, in the hope of finding compounds with a definite margin between these doses.

Similarly, as in Spiegel's[4] previous experiments, the effect upon the threshold for production of convulsions by electric stimulation was determined. This method permits one to ascertain the effect of anticonvulsant agents more unequivocally than the study of antagonistic effects against convulsant drugs such as metrazol (Selye[6]) or cocaine (Aird[7]), since such an antagonism may be due, at least partly, to other factors such as decrease of absorption or of permeability of the cerebral capillaries. The existence of such mechanisms is indicated by the fact that Aird found a protective influence of desoxycorticosterone acetate against cocaine convulsions in doses that are unable to alter the threshold of the brain for production of convulsions by electric stimulation.

We were able to study twenty-nine steroids.*

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METHOD

Definite anticonvulsant effects were observed by Spiegel[4] on white female rats. These animals were also employed in Selye's[3] studies of hypnotic effects. In this study, therefore, we used almost exclusively white female rats of from 100 to 150 grams body weight (over 200 animals). Since male rats were resistant even to quantities far beyond the doses effective in female rats and our supply of steroids was limited, we tested these compounds in male rats only occasionally.

The convulsion threshold was determined by using the previously described method of electrical stimulation with the skull intact (Spiegel[8]). The alternating current from the 110-volt, 60-cycle line is reduced by a Variac transformer to the desired voltage (usually 10 volts). After leaving the transformer the alternating current is closed and opened by a relay, the magnet of which is activated by the discharge of condensers (from 1 to 20 μ F). Since the duration of the activation of the magnet is proportional to the capacity of the condenser, the resistance of the relay circuit being constant, the duration of the stimulation can be varied. The electrodes are placed in the conjunctival sacs. At a certain voltage the duration of the stimulation is increased, step by step, until typical tonic-clonic epileptiform convulsions are elicited. The threshold values are calculated in milliampere seconds since the voltage and the duration of the stimulation are known, and the resistance can be determined on a Wheatstone bridge, the electrodes remaining on the same place as during the stimulation.

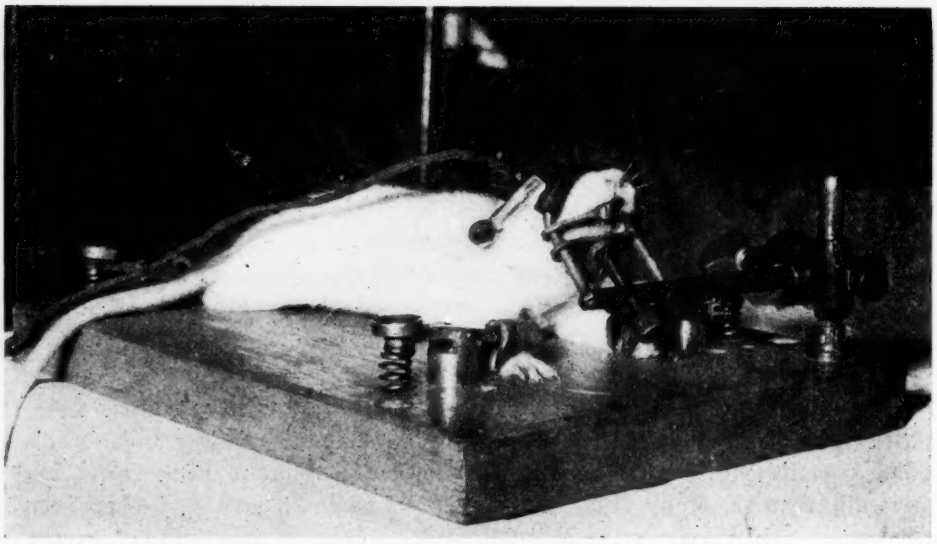


Fig. 1.—Rat board and head holder. Note electrode in right conjunctival sac.

The fixation of the head, application of the stimulating electrodes to the eyeballs, and observation of the convulsions were facilitated by the use of an especially designed head holder (Fig. 1) that fastens the snout, leaving the rest of the head and the eyeballs free. In rats it was of course necessary to apply much smaller electrodes than in rabbits or cats. The electrodes used were slightly concave silver disks with a diameter of 5 mm., which were fitted over the eyeballs. They were held in the conjunctival sacs by small clamps that kept the lids tightly together. With these electrodes the resistance in the circuit usually was between 700 and 1200 ohms. As a rule, at 10 volts a flow of the current from 0.1 to 0.7 seconds sufficed in untreated animals for production of convulsions.

In all experiments the threshold was determined in milliampere seconds, and the type (extent, localization) and duration of the convulsions were recorded. In order to ascertain the variability of the threshold, at least three tests were made preceding the injection at intervals of from two to three days, and only such animals were used for the injection in which the threshold varied by no more than 30 per cent. The substance under study was applied, as a rule in oily solution, by intraperitoneal injection; occasionally, intramuscular injection of solutions in propylene glycol was used. The convulsion threshold was then determined from one-half to one hour following the injection. The ratio A/B of the convulsion threshold after injection (A) to the average before injection (B) served as a measure of the anticonvulsant effect.

RESULTS

A substance was considered ineffective, if the ratio A/B was from 1.0 to 1.3 and only slightly effective if this ratio was smaller than 1.5; it was considered anticonvulsant, if this ratio was above 1.5. Applying these criteria, the substances were divided into two groups. In Table I are summarized those which were ineffective or only slightly effective even in relatively large doses.

TABLE I. COMPOUNDS WITH NO, OR ONLY SLIGHT, ANTICONVULSANT EFFECT*

COMPOUND	DOSE IN MG. PER 100 GM. BODY WEIGHT	CONVULSION THRESHOLD AFTER (A): BEFORE (B) INJECTION
Cholesterol (30 mg. per cubic centimeter olive oil injected in rabbits)	18 to 21 (3 to 5 daily injections)	0.5 to 1.3
Allocholesterol (St)	20 and 50	1
Cholesteryl bromide (St)	20 and 50	1
Epicholestanol (St)	50	1
Stigmasterol (St)	20 and 50	1
Stigmasteryl acetate (St)	20 and 50	1
α-Spinasteryl acetate (St)	20 and 50	1
Ergosterol (St)	20 and 40	1
Ergosteryl acetate (St)	20 and 50	1
α-Ergostenyl acetate (St)	50	1
Dehydrocholic acid (St)	50	1
Desoxycholic acid (St)	20	1
	50	1
Δ^5-3-Acetoxycholenic acid (St)	50	0.7 to 1.2
Sarsasapogenin acetate (K)	20 and 50	1
Pseudo-sarsasapogenin acetate (K)	20	1
	50	1.2
Diosgenin acetate (K)	20 and 50	1
Pseudo-diosgenin acetate (K)	20 and 50	1
α-Estradiol benzoate (Progynon B; HS)	10,000 rat units	1.3
1.66 mg. per cubic centimeter sesame oil (10,000 rat units)	20,000 rat units	1.1
5 mg. per cubic centimeter (30,000 rat units)	4-8 mg.	1
Theelin in oil (K) 1 c.c. = 10,000 I.U.	10,000 and 20,000 I.U.	1.3
6 (α) Acetoxy-progesterone (E) 10 mg. per cubic centimeter; amorphous modification	5 and 10	1
	20	1 to 1.1
Etio-cholan-3ß-ol-17-one acetate (K)	20 and 40	1
	50	1.0 to 1.6
5-Pregnen-3ß-ol-20-one acetate (K)	20	1
	30	1.3
	50	1.4
5, 16-Pregnadien-3ß-ol-20-one acetate (K)	15	1
	20	1.0 to 1.3
	30	1
Stilbesterol 13 mg. per cubic centimeter olive oil	10	0.9 to 1.1
	20	0.7 to 1.0
20 mg. per cubic centimeter olive oil	30	1

*If not otherwise indicated, all substances were dissolved in olive oil, 20 mg. per cubic centimeter and injected intraperitoneally in white female rats. The source of the material is marked as follows: *St*, Dr. H. E. Stavely; *K*, Dr. O. Kamm; *HS*, Dr. E. Henderson and Dr. E. Schwenk; *E*, Dr. M. Ehrenstein.

In Table II are shown the steroids with a definite anticonvulsant effect manifested by an increase of A/B above 1.5 and/or by a decrease of the duration of the convulsions, the reaction being reduced in some instances (for example, after injections of progesterone) to single twitches. In Table I cholesterol also is included. We have already found in previous studies[9] that injection or feeding of this compound fails to raise the convulsion threshold. Likewise in Table I are included compounds similar to cholesterol such as allocholesterol, cholesteryl bromide, epicholestanol, phytosterols (stigmasterol, stigmasteryl acetate, α-spinasteryl-acetate) and mycosterols (ergosterol, ergosteryl acetate, α-ergostenyl acetate), further bile acids, and derivatives (dehydrocholic acid, desoxycholic acid, Δ^5-3-acetoxy-cholenic acid), and digitalis sapogenins (sarsasapogenin acetate, pseudo-sarsasapogenin acetate, diosgenin acetate, pseudo-diosgenin acetate). We included in Table I some steroids with hormonal properties and

TABLE II. COMPOUNDS WITH DEFINITE ANTICONVULSANT EFFECT

COMPOUND	DOSE IN MG. PER 100 GM. BODY WEIGHT	CONVULSION THRESHOLD AFTER INJECTION (A): THRESHOLD BEFORE INJECTION (B)
Testosterone (25 mg. per cubic centimeter sesame oil, HS)	17	1 to 1.2
	18	1.5
Thirteen animals tested	19	1 to 2
	21	1.7 only very mild convulsions
	25	2
	26	1.7 in large animal (169 Gm.)
Androstenedione (20 mg. per cubic centimeter olive oil, HS, St)	5	1.2 to 1.5
	7	1.5 to >2.0
Twenty-seven animals tested	8	>2
	10	1.0 to 3.0
	11	>3
	12	3 to >4
	15	2 to >4
	20	3
Dehydroandrosterone (20 mg. per cubic centimeter olive oil, HS, St)	8	1.1 to 2.5
	10	1.8 to 2.5
Thirteen animals tested	15	2.4
	20	1.6 to 3.0
	30	1.6 to 4.0
	40	2.4
Progesterone (12 mg. per cubic centimeter sesame oil, HS)	5	1.0
	6.5	Ratio could not be determined exactly because only rudimentary twitchings were elicited
Ten animals tested	8	Same
Acetoxypregnenolone (4 and 5 mg. per cubic centimeter sesame oil,* (HS)	2.0 and 2.5	1.0
	3	1.0 to 1.7
Thirty-five animals tested	4	2.1 to 3.7
	8	2.0 to 3.0
Desoxycorticosterone acetate (5 mg. per cubic centimeter cod-liver oil, HS)	20	1
	23	1.2
Eight animals tested	25	1 to 2.0
	27 and 28	>1.5; marked reduction of duration of convulsions
(15 mg. per cubic centimeter sesame oil, HS)	1 to 2	1.0 to 1.2
Eleven animals tested	2.5	1.7
	3.2	1.2 marked reduction of duration
	4.3	Unexcitable
	5	2.0 (duration reduced)
(15 mg. per cubic centimeter in male rats)		
Five animals tested		
(Weight, from 200 to 300 Gm.)	8 to 40	0.6 to 1
(Weight, from 150 to 170 Gm.)	35 to 48	No definite convulsions (current produced only brief single twitches)

*Four milligrams per cubic centimeter prepared by us and 5 mg. per cubic centimeter prepared by Schering Corporation.

parent substances or derivatives that had no, or only a small, anticonvulsant effect in the doses at our disposal. This group comprises estrogenic hormones (α-estradiol benzoate, theelin), the amorphous modification of 6(α) acetoxyprogesterone, etiocholan-3β-ol-17-one acetate, 5-pregnen-3β-ol-20-one acetate, and 5, 16-pregnadien-3β-ol-20-one acetate. Finally, in Table I is contained stilbestrol that chemically does not belong in this group, but has folliculoid effects.

The steroids that manifested definite anticonvulsant effects as summarized in Table II belong to the groups of sex hormones and adrenal substances. It should be emphasized that as a rule anticonvulsant effects could be obtained with much smaller amounts, if high concentration rather than low concentrations of a compound were injected; for example, anticonvulsant effects were obtained with desoxycorticosterone acetate in a solution of low concentration (5 mg. per cubic centimeter) only if doses above 23 mg. per 100 Gm. body weight were injected, while from 2.5 to 3.2 mg. per 100 Gm. sufficed if a more concentrated solution (15 mg. per cubic centimeter) was used, apparently because an efficient blood concentration was more easily reached in the latter case.* A further factor is the size (age) of the animal. In general, in rats weighing 100 Gm. or less, smaller relative doses (per 100 Gm. body weight) were sufficient to obtain an anticonvulsant effect than in rats weighing 150 Gm. or above. The sexual factor has already been mentioned. It is illustrated by the experiments with desoxycorticosterone acetate. In doses from 8 to 40 mg. per 100 Gm., this compound in concentrated solution produced in male rats weighing from 200 to 300 Gm. no change or even a slight increase of the convulsive reactivity; in smaller animals weighing from 150 to 170 Gm., a reduction of the reactivity was obtained with doses of from 35 to 48 mg.; this is about ten times the dose that was sufficient in female rats for such an effect.

In estimating the efficiency of an anticonvulsant substance, it seems desirable not only to determine its effect upon the convulsion threshold, but also upon the margin between anticonvulsant effect on the one hand and hypnotic effect on the other. In Table III these margins are shown for the steroids that we found to be distinctly anticonvulsant. In determining the minimum hypnotic dose, signs of depression of the activity of the central nervous system were looked for, such as reduction of spontaneous movements, ataxia, impairment of the righting reflexes, and reduction of the reactions to painful stimuli.

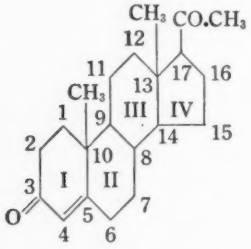
TABLE III. MARGIN BETWEEN MINIMUM ANTICONVULSANT AND HYPNOTIC EFFECT

COMPOUND	MINIMUM ANTICONVULSANT DOSE (MG.)	MINIMUM HYPNOTIC DOSE (MG.)
Testosterone	18 to 19	18 to 19
Androstenedione	7 to 10	10 to 12
Dehydroandrosterone	8 to 10	12 to 15
Progesterone	6.5	6.5
Acetoxypregnenolone	3 to 4	4 to 5
Desoxycorticosterone acetate		
5 mg. per cubic centimeter	25 to 26	25 to 26
15 mg. per cubic centimeter	2.5 to 3	2.5 to 3

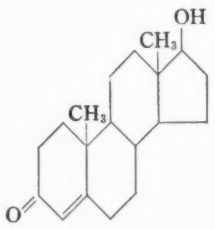
*It is, of course, of paramount importance to overcome the poor solubility of many of these substances. Steroids that were definitely effective in clear solution proved ineffective if only coarser dispersions were injected. Shaking the suspensions for several hours, or even for days, in a shaking machine helped only slightly. Gently heating on a water bath resulted in much better solutions but seemed in some instances, for example, acetoxypregnenolone, to impair the effectiveness of the substance under study. In such a case the steroid was dissolved in small amounts of chloroform; this solution was mixed with the oil and the chloroform evaporated by keeping the solution for several days in a vacuum at room temperature. In control experiments, such chloroform-oil mixtures, without the steroid under study, were kept under the same conditions in the vacuum; intraperitoneal injections of corresponding quantities of these control solutions were ineffective.

It can be seen from Table III that the anticonvulsant dose lies rather close to, or is identical with, the hypnotic dose for testosterone, progesterone, and desoxycorticosterone acetate, while a definite margin between these doses exists for androstenedione, dehydroandrosterone, and acetoxypregnenolone.

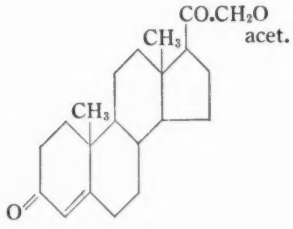
Regarding the chemical structure of those compounds that revealed definite anticonvulsant effects, we deal with tetracyclic derivatives of cyclopentenophenanthrene (see Sobotka[10]), having only one double bond in the first ring except dehydroandrosterone and acetoxypregnenolone, which are unsaturated in the 5, 6 position (see below). At positions 10 and 13 all have angular methyl groups; at C_3 all are oxygenated, except acetoxypregnenolone and dehydroandrosterone, which are here hydroxylated. The only other side chain is at position 17; here are found most of the differences, testosterone having an OH group, androstenedione and dehydroandrosterone, an O; progesterone, a $CO \cdot CH_3$;



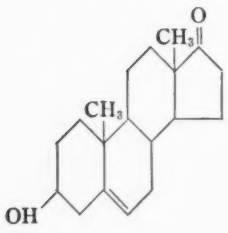
Progesterone



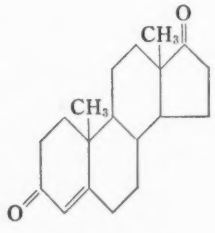
Testosterone



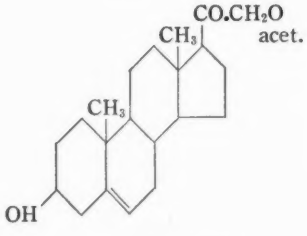
Desoxycorticosterone acetate



Dehydroandrosterone



Androstenedione



Acetoxypregnenolone

acetoxypregnenolone and desoxycorticosterone acetate, $CO.CH_2O$ acetate. A comparison between testosterone and androstenedione seems to indicate that ketogenic oxygen at C_{17} is favorable to an anticonvulsant effect. However, we do not wish to draw definite conclusions in this respect before testing further compounds with similar structure.

SUMMARY

Among twenty-nine steroids tested, desoxycorticosterone acetate, progesterone, testosterone, acetoxypregnenolone, androstenedione, and dehydroandrosterone were able to increase the threshold of electrically induced convulsions. A definite margin between anticonvulsant and hypnotic dose was found for the latter three substances only.

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CERTAIN ASPECTS OF THE BRONCHIAL REFLEXES OBTAINED BY STIMULATION OF THE NASOPHARYNX

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IT HAS been known for many years that stimulation of the nasopharynx produces reflex bronchoconstriction.[1-5] The pathway of this reflex has been believed to be afferent via the sensory fibers of the trigeminal nerve and efferent down the vagus. It has been demonstrated, however, that atropine is incapable of entirely blocking the reflex cardiac slowing from nasal stimulation,[6] and a later report indicates that atropine is also incapable of blocking the coronary constriction induced by nasal stimulation.[7] Both of these effects have, therefore, been assumed to be due in great part to a "sympathetic inhibition" of tone. In view of these findings we decided to investigate further the nature of the nasobronchial reflex.

All the experiments were done on decerebrated dogs. It is necessary to obviate the influence of higher centers which inhibit reflexes. The more commonly used anesthetic agents in adequate dosage are prone to depress or abolish reflexes. The animals were rendered decerebrate by exposing the cortex, dissecting down to the region of the red nucleus and severing the brain stem in this region. The dogs thus rendered decerebrate displayed typical spasticity, tremors, clasp-knife rigidity, etc. The method of recording bronchial constriction was by means of a very small inflated rubber balloon inserted into one of the small bronchi and attached to a tambour and writing lever. During the production of these reflexes an open pneumothorax was established on the same side as the balloon. This was done to so equalize intrabronchial and intrathoracic pressure that any change in the force of respiration would not affect the pressure on the balloon in the bronchus. Several experiments were done, however, without the open pneumothorax, which showed essentially the same results as the others. To validate this method we injected several well-known bronchoconstricting agents (acetylcholine, histamine, mecholyl) and stimulated electrically the peripheral end of the cut vagus nerve (Fig. 1). In every instance we noted a

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distinct rise in the base line. Bronchodilators, such as epinephrine, caused rather marked falls in the base line (Fig. 2). It is appreciated, of course, that our method will record only changes produced in the bronchi and not in the bronchioles or respiratory bronchioles. Since the bronchi act, in so far as is known, as do the smaller bronchioles, we feel the method to be valid.

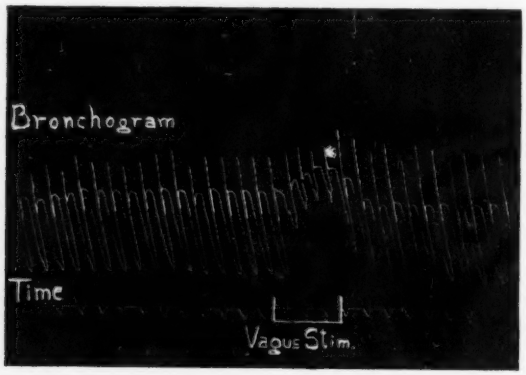

Fig. 1.—Effect of electrical stimulation upon the bronchogram.

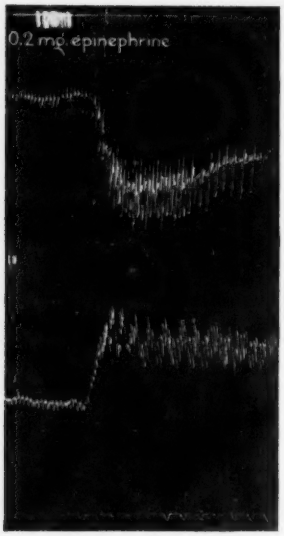

Fig. 2.—Effect of 0.2 mg. of epinephrine on the bronchogram shown in the upper tracing, with the blood pressure effect shown in the lower tracing.

As a means of stimulating the nasal mucosa, we have used cold air blown directly into the nostril or cold water, ethyl chloride, and chloroform vapor blown into a nostril. As a general rule, if any of these stimulating agents was effective, all of them were.

The general procedure was to elicit this reflex, then administer a substantial dose of atropine (up to 10.0 mg.) or cut the vagi and repeat the nasal stimulation. Then ergotamine tartrate was administered (from 0.5 to 1.0 mg.) and the nasal stimulus again applied. The results are given in Table I.

It can be seen that in the majority of animals neither atropine nor vagotomy was entirely successful in abolishing this reflexly induced bronchoconstriction (Figs. 3, 4, and 5). Since atropine in large doses consistently paralyzes the vagus, it is apparent that this nasobronchial reflex is not mediated entirely by the vagus nerve. As the observed bronchoconstriction is undoubtedly reflex in

TABLE I. EFFECT OF AGENTS OR PROCEDURES ON REFLEX BRONCHOCONSTRICTION INDUCED BY NASAL STIMULATION

	ATROPINE	ERGOTAMINE	VAGOTOMY	ATROPINE AND ERGOTAMINE
No effect	3	1	2	0
Effect diminished	2	2	3	0
Effect abolished	0	2	1	5

nature, the only other nervous mechanism capable of producing this change is a reduction in the tone of the sympathetic nerves innervating the bronchi. Very powerful bronchodilator nerves have been demonstrated by Dixon and Ransom,[8] so such a phenomenon is not unknown. Since ergotamine is capable of blocking sympathetic motor nerves,[9] then, with their activity abolished, a dependent reflex must likewise be abolished. We have found this to be true.

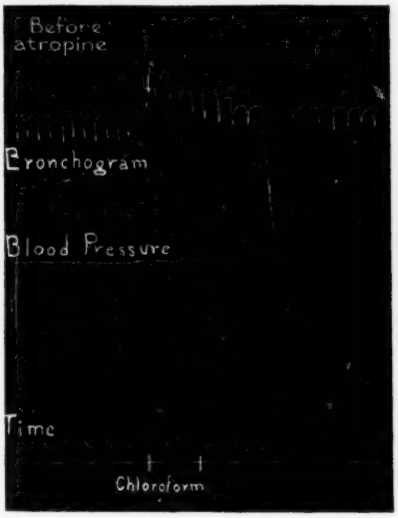

Fig. 3.

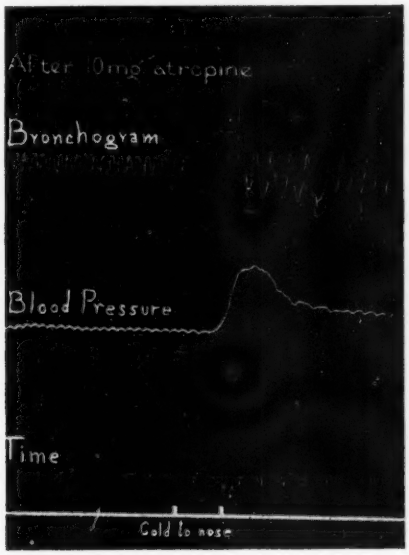

Fig. 4.

Fig. 3.—Effect of stimulation of the nasal mucous membrane by chloroform vapor before the use of atropine.

Fig. 4.—Effect of stimulation of the nasal mucous membrane by chloroform after 5 mg. of atropine.

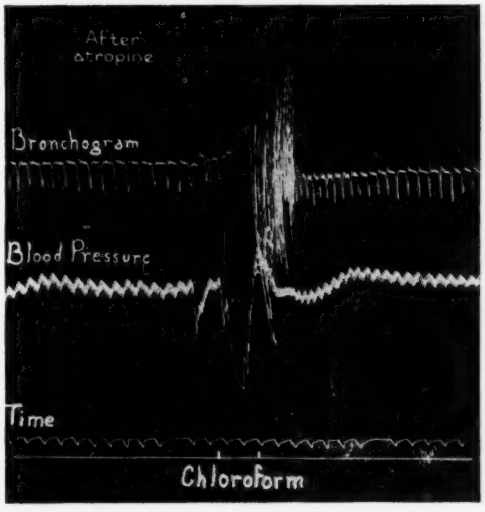

Fig. 5.—Effect of stimulation of the nasal mucous membrane by ethyl chloride after 10 mg. of atropine. Stimulation by ice water gives similar results.

If this work is valid and can be transposed to human subjects, then an interesting question is raised. For many years, the results of atropine therapy in the treatment of asthma have been very disappointing. In particular, atropine therapy of the asthmas resulting from exposure to cold air has been unsatisfactory. The explanation has been suggested that this is an allergy in which exposure to cold results in the local release of histamine, an extraordinarily powerful bronchoconstricting agent. It is possible that this mechanism might well be augmented by reflex constriction and, if this is a result of sympathetic inhibition, the ineffectiveness of atropine is understandable. Likewise, the efficacy of epinephrine and other sympathomimetic amines is understandable. It is well to keep this reflex in mind in breathing cold air through the nostrils at altitudes.

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PROGRESS

RH FACTORS IN CLINICAL MEDICINE*

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ALMOST every important scientific discovery has a long historical background. Intimately associated with the history of the Rh blood factors is the name of the late Dr. Karl Landsteiner. Dr. Landsteiner is correctly known as the father of blood grouping, and in 1930 he was awarded the Nobel Prize in Medicine primarily for his discovery of human blood groups[1, 2] which made possible the safe transfusion of blood. Early in his studies Dr. Landsteiner conceived the existence of numerous serologic differences in human blood which might some day make possible the individual identification of human beings based on their blood reactions in a manner somewhat similar to the use of fingerprinting. The discovery of the subgroups in Groups A and AB by von Dungern and Hirszfeld[3] in 1910 and of the properties M, N, and P in 1928 by Landsteiner and Levine[4] increased the number of subdivisions of human blood from four to thirty-six; that was the status of the subject when I began my first studies on blood grouping in collaboration with Dr. S. H. Polayes in 1929. As a result of my interest in blood grouping, I became intimately acquainted with Dr. Landsteiner. Our first meetings soon blossomed into a close friendship which continued until his untimely death in 1943, depriving me of an irreplaceable friend and teacher and the medical world of one of its leading scientists.

Some of the earlier investigations on additional factors in human blood besides A, B, M, N, and P did yield some findings supporting Landsteiner's idea of the existence of numerous individual differences in human blood.[5] However, attempts to reproduce these findings were not consistently successful because no satisfactory method could be devised for obtaining the necessary antisera. In 1937 I became interested in the evolution of the agglutinogens M and N, and when my preliminary finding proved interesting,[6] I continued the work in collaboration with Dr. Landsteiner. In the blood of rhesus monkeys we detected the presence of M-like agglutinogens and also found that the injection of the blood of rhesus monkeys into rabbits stimulated the production of anti-M agglutinins.[7] Previously it had been found by Schiff that injections of sheep blood into rabbits caused the formation of anti-A agglutinins and hemolysins. It therefore occurred to us that by continuing this line of research, namely, the use of animal blood instead of human blood for preparing our antisera, we might discover new factors in human blood. Indeed, we did find[8] that some of our antisera for rhesus monkey blood contained an agglutinin reacting with the blood cells of 85 per cent of the white population independently of the blood groups or M, N, and P factors. The new factor detected by these rhesus anti-

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sera we later designated as Rh to indicate the manner in which it had first been discovered.

We did not immediately report our findings because we wished to perfect the method of producing the antisera. When, in 1939, Dr. H. Raymond Peters and I encountered three cases of hemolysis following transfusions of blood of the homologous group, which were proved to be due to isoimmunization to the rhesus factor,[9] it became evident that the new blood factor was of clinical importance. This is the reason why the Rh factor found by Landsteiner and myself in 1937 was first announced by us in January, 1940.[8]

As already mentioned, the blood cells of 85 per cent of all white individuals are agglutinated by the antirhesus sera. Such individuals are said to be Rh positive, while the remaining 15 per cent are said to be Rh negative. These two types of blood are hereditarily determined like the four blood groups, the Rh factor being transmitted as a single Mendelian dominant[10] by a pair of allelic genes, *Rh* and *rh*. Since every individual possesses a pair of genes from every series of allelic genes, one gene being derived from the mother and the other from the father, there are three genotypes possible. Rh-negative individuals belong to genotype *rhrh* and are always homozygous; Rh-positive individuals may either be homozygous (genotype *RhRh*) or heterozygous (genotype *Rhrh*). Therefore, two Rh-negative parents can only have Rh-negative children. If one parent is Rh negative and the other is Rh positive, the children will all be Rh positive if the Rh-positive parent is homozygous, or one-half the children will be Rh positive and one-half Rh negative if the Rh-positive parent is heterozygous. When both parents are Rh positive, all the children will be Rh positive, except when the parents are both heterozygous, in which case one-fourth of the children will be Rh negative.[11]

Natural Rh isoantibodies have never been demonstrated in human serum. Therefore, Rh-negative individuals can safely be transfused with Rh-positive blood provided that they have not become sensitized to the Rh factor as a result of previous exposure to the Rh antigen. The situation with regard to Rh in relation to blood transfusion is therefore different from that which exists for the blood groups, in which every individual (except in infancy) has agglutinins in his or her serum for those agglutinogens (A, B or both) which are lacking from the erythrocytes, so that even a first transfusion of an incompatible blood may cause a hemolytic reaction. The situation is also different from that which exists for the properties M, N, and P. Natural antibodies for M and P have been demonstrated in a number of human sera[12-14] and in a few instances the transfusion of blood containing properties M and P has stimulated the production of anti-M and anti-P immune antibodies.[15, 16, 17] These instances are nevertheless extremely rare and no instance has yet been encountered of natural or immune isoagglutinins for N, so that it is evident that properties M, N, and P only rarely have to be considered when donors are selected for blood transfusion. The clinical importance of property Rh depends on the fact that Rh is a far better antigen in man than M, N, and P, as evidenced by the observation that as many as 90 per cent of cases of intragroup transfusion hemolysis can be traced to the Rh factor.[18]

Rh-negative individuals may become sensitized to Rh antigen in one of two ways: (1) sensitization may result from a transfusion of Rh-positive blood or (2) in women, sensitization may result from pregnancy with an Rh-positive fetus. Individuals sensitized by either of these two methods may have a serious hemolytic reaction if transfused with Rh-positive blood.[9,18] Ideally,

therefore, every patient who is to be given a blood transfusion should be tested for the Rh factor as well as the blood group, and Rh-negative patients should only be given Rh-negative blood. Under battle conditions, of course, this precaution is hardly ever necessary because the casualties are mostly men who have never had a previous blood transfusion; in fact, it is undesirable because insistence on Rh tests would unduly delay a lifesaving procedure. In civilian hospitals such tests should be insisted on for all patients requiring a series of transfusions, and, if possible, for all women patients, particularly during the child-bearing age. It is important to mention at the onset that not every Rh-negative person receiving transfusions of Rh-positive blood or bearing an Rh-positive fetus becomes sensitized to the Rh factor. Individuals differ in the ease with which they can be sensitized, probably depending on some hereditary constitutional quality,[19] so that on the average only one in about twenty-five Rh-negative persons exposed to the Rh antigen becomes sensitized. This explains the relative infrequency of intragroup transfusion hemolysis due to the Rh factor, so that such cases were uncommon even before 1940 when the existence of the Rh factor was still unknown and tests for Rh were not carried out before transfusion.

Soon after the importance of the Rh factor as a cause of intragroup transfusion hemolysis was pointed out by Wiener and Peters, Levine and his collaborators[20] noticed that intragroup transfusion reactions occurred with exceptional frequency in women who had had stillbirths or infants with erythroblastosis fetalis. This suggested that isoimmunization in pregnancy might be the basis of the latter disease, which Diamond, Blackfan, and Baty[21] had previously shown to comprise a number of syndromes, namely, icterus gravis neonatorum, hemolytic anemia of the newborn, hydrops fetalis, and certain unexplained stillbirths. According to the theory of Levine and associates,[22] an Rh-negative woman bearing an Rh-positive fetus becomes sensitized to the Rh factor, the Rh antibodies produced by her pass through the placenta into the fetal blood circulation and combine with the red cells, giving rise to one or another manifestation of the disease. In support of this theory may be cited Levine's observation that 90 per cent of the erythroblastotic infants have mothers who are Rh negative, while, as already mentioned, only 15 per cent of individuals in the general population are Rh negative. Again, in about one-half of the cases, anti-Rh agglutinins could be demonstrated in the maternal serum; this proved that the mothers had been sensitized to the Rh factor. In view of this evidence concerning the nature of the disease, the name has been changed from erythroblastosis to hemolytic disease of the fetus and newborn or congenital hemolytic disease; the latter name will be used henceforth in this lecture.

While the mating of Rh-negative women with Rh-positive husbands occurs in one of ten marriages, congenital hemolytic disease affects only one of about 250 newborn infants. This suggests that only about one in twenty-five Rh-negative women becomes sensitized when bearing an Rh-positive fetus, just as in sensitization by blood transfusion. The firstborn is rarely affected, because it takes at least one pregnancy and sometimes more before a sufficient degree of sensitization develops. In most of the rare cases where the firstborn was affected, a careful history revealed that the woman had previously received a transfusion of Rh-positive blood.[23, 24] This serves to emphasize the importance of performing Rh tests before giving blood transfusions to women in the child-bearing age, and even to young girls, because such a transfusion could ruin a woman's chance to have a normal child.

While the theory of Levine and co-workers accounts for many of the essential facts in the pathogenesis of congential hemolytic disease, a number of apparent paradoxes remain to be explained. Since the intact villus is impermeable to erythrocytes, it is not clear how the fetal Rh antigen gains access to the maternal circulation. There is no convincing evidence that Rh antigen exists in soluble form in body fluids,* as is the case for the A and B factors, despite the assertions of certain workers.[25, 25a] The most plausible explanation is that during labor and delivery, villi become detached and enter the maternal circulation, carrying along enough fetal red cells to incite antibody formation by those women who are constitutionally predisposed. Autopsies on women who have died in childbirth or shortly thereafter have actually revealed the presence of Langerhans giant cells and villi in the lung bed. At the same time, this would explain why congenital hemolytic disease rarely affects the firstborn unless the mother has been previously sensitized by blood transfusion. It is difficult to reconcile Levine's theory with the lack of correlation between the titer of anti-Rh agglutinins in the maternal serum and the severity of the disease in the infants;[26, 27] thus in some of the most severe cases of congenital hemolytic disease no Rh agglutinins are detectable in the maternal serum; on the other hand, some Rh-negative women with anti-Rh agglutinins in their serum have given birth to apparently normal Rh-positive infants. The occasional occurrence of hemolytic disease when the mother is Rh positive has been attributed to subtypes of Rh and to the Hr factor, as will be explained later, and some cases have also been attributed to isoimmunization to the A-B blood group factors. With regard to the common blood groups, it is not clear why the A-B factors so rarely cause trouble, considering that the maternal serum frequently contains natural isoagglutinins anti-A and anti-B, and A and B are far more potent antigens than Rh. The suggestion that nonsecretory infants are the only ones affected is improbable *a priori* because as many as 15 per cent of all individuals are nonsecretors, while these cases are uncommon; besides, in two cases recently seen by me both infants proved to be secretors. Another puzzling feature of congenital hemolytic disease not explained by Levine's theory is why infants frequently appear perfectly normal when born, yet within a few hours or days severe jaundice and anemia develop.

These paradoxes have been explained by the recent discovery[28, 29] that more than one variety of Rh antibody is produced by individuals sensitized to the Rh factor. In addition to anti-Rh agglutinins, antibodies designated "blocking antibodies" and "glutinins" are produced; these antibodies are far more important than the agglutinins in the causation of congenital hemolytic disease. The blocking antibodies and glutinins were not found previously because special techniques had to be devised for their detection, as will now be explained.

TESTS FOR RH SENSITIZATION

Agglutination Test.[30]—In the agglutination test for Rh sensitization, a drop of the patient's serum is mixed with a drop of a 2 per cent saline suspension of Group O Rh-positive cells in a small test tube and the mixture incubated in a water bath at body temperature for from thirty to sixty minutes. The reactions can usually be judged from the appearance of the sediment at the bottom of the tube (see Fig. 1). The sediment is then gently dislodged by twisting the tube and the mixture is examined for the presence or absence of

*If the Rh antigens are actually present, their concentration must be far smaller than that of the A-B-O antigens.

agglutination. In positive reactions the red cells are clumped together in large masses usually visible to the naked eye, while in negative reactions the mixture remains homogeneous with each red cell separate and distinct, as proved by microscopic examination. When the patient's serum contains only very small

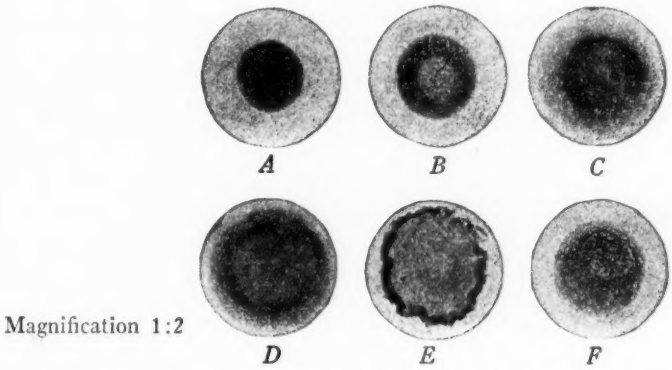

Fig. 1.—Sedimentive method of testing for the Rh factor (magnification, 1:2). *a* and *b*, negative reactions; the inner light disc in *b*, is due to slight convexity in the bottom of the tube. *c*, Faintly positive reaction. *d*, Weak positive reaction. *e*, and *f*, Typical positive reactions. (From Landsteiner and Wiener: J. Exper. Med. 74: 312, 1941.)

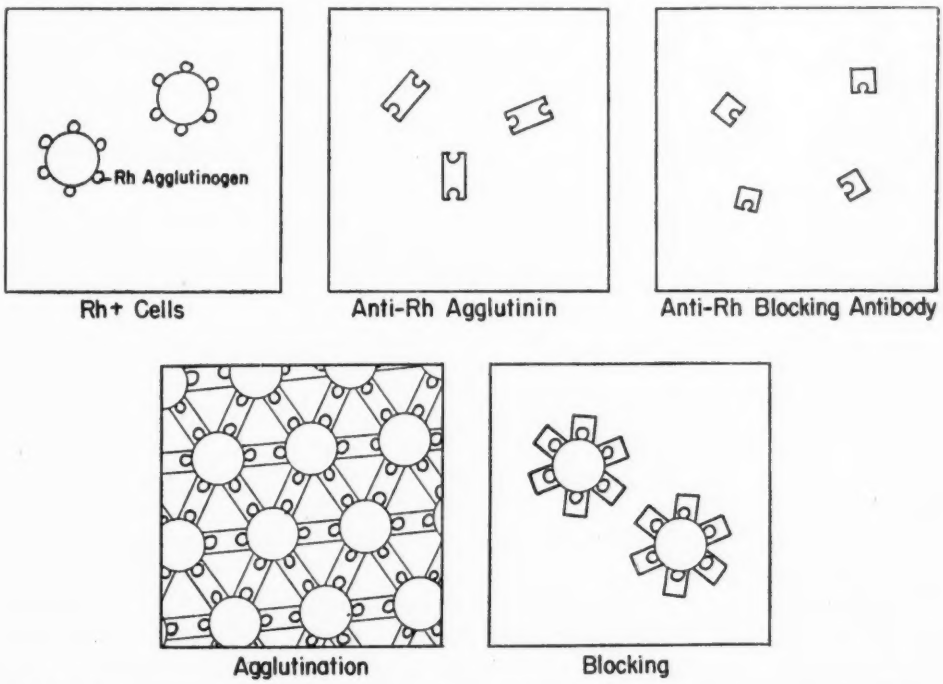

Fig. 2.—Diagrammatic representation of Rh agglutination and blocking reactions. (From Wiener, A. S.: Am. J. Clin. Path. 15: 106, 1945.)

quantities of Rh agglutinin, centrifugation may be necessary to bring out the reaction. When enough agglutinin is present, on the other hand, strong clumping will occur even in tests with the patient's serum diluted with saline solution, and in tests on slides as well as in tubes.

According to our present concept, Rh agglutinins, like other antibodies in general, are modified serum globulins. Moreover, Rh agglutinins like other agglutinins are presumably polyvalent (probably bivalent) so that each anti-

body molecule has more than one specific combining group for the corresponding Rh hapten. Since the red cells presumably have numerous Rh haptens distributed over their surfaces, when agglutinin and agglutinogen combine, clumping results from the formation of a latticework, in accordance with Marrack's hypothesis (see Fig. 2). This[31a, 31b] concept of the nature of Rh agglutinins is useful in explaining the pathogenesis of congenital hemolytic disease, as will be pointed out later on.

Blocking Test.[28, 32, 33]—In search for an explanation of cases of congenital hemolytic disease which were typical except that the maternal serum contained no demonstrable Rh agglutinins, it occurred to me that Rh antibodies might be present but of a special variety incapable of bringing about agglutination. If such antibodies were present and combined with the Rh-positive red cells, then the cells might be expected to lose their capacity to agglutinate upon the further addition of anti-Rh agglutinating serum. In preliminary experiments carried out by me in 1941 some encouraging results were obtained, but final proof of the existence of such special antibodies, designated as Rh blocking antibodies, was not obtained until 1944 when larger quantities of potent human anti-Rh serum became available for these tests.[28]

In the test, a drop of the patient's serum is first mixed with a drop of a suspension of Group O Rh-positive red cells and incubated in the water bath. In the absence of Rh agglutinins, no clumping should be evident at this stage. Then a drop of a good human anti-Rh agglutinating serum is added, and the mixture is reincubated in the water bath. If the patient's serum does not contain any Rh antibodies, strong clumping should be evident; on the other hand, if the patient's serum contains Rh blocking antibodies, the red cells remain unagglutinated or the clumping is distinctly weakened.

I have postulated that Rh blocking antibodies, in contrast to Rh agglutinins, are univalent.[28] This would account for the ability of these antibodies to combine with Rh agglutinogen and to block the combining sites on the surface of the erythrocytes, but without clumping them (see Fig. 2). If this concept is correct, it seems reasonable to conclude that Rh blocking antibodies are comprised of smaller molecules than Rh agglutinins. Therefore, Rh blocking antibodies should be capable of traversing the placental barrier into the fetal circulation more readily and earlier in pregnancy than Rh agglutinins. This is supported by the clinical observation that the presence of Rh blocking antibodies is of more serious prognostic import than the presence of Rh agglutinins without blocking antibodies; for example, when blocking antibodies are present, stillbirths are more likely to result.

The discovery of the Rh blocking antibodies has also served to explain certain intragroup hemolytic transfusion reactions. For example, I have encountered many cases of severe transfusion hemolysis in male patients due to Rh isoimmunization by repeated transfusions. In two such cases, the patients were Rh negative, but their sera contained no anti-Rh agglutinins, and in the usual compatibility test the sera of the patients failed to agglutinate the blood suspensions of the donors whose blood caused the reactions. However, the blocking tests were positive in both cases and showed the presence of Rh antibodies of high titer.

Conglutination Test.[29]—The discovery of the Rh blocking antibodies explains why the classic cross-matching tests have been inadequate to detect all cases of intragroup incompatibility. By slightly modifying the technique of the compatibility test, it is now possible to avoid this pitfall.

The new test for Rh sensitization, designated as the conglutination test, differs from the agglutination test in that it occurs in two stages instead of one and because a third component besides the Rh antigen and its specific antibody must be present in the mixture in order for clumping to occur. The third component, like complement in serologic hemolysis, is absorbed onto the specifically sensitized red cells and then causes them to stick together. However, this third component is distinct from complement. It resists heating at 60° C. and is a colloidal constituent in the plasma probably identical with the so-called X protein, which is a large molecular complex of albumin, globulin, and phospholipid. The Rh antibody participating in the conglutination reaction is univalent and is best referred to as Rh glutinin, with the understanding that it is still uncertain whether or not glutinin is identical with blocking antibody.

The difference between Rh agglutination and Rh conglutination can be summarized as follows:

$$\left.\begin{matrix}\text{Rh-positive}\\ \text{red cells}\end{matrix}\right\} + \left\{\begin{matrix}\text{Bivalent Rh antibodies}\\ \text{(Agglutinins)}\end{matrix}\right\} \rightarrow \text{Agglutination}$$

$$\left.\begin{matrix}\text{Rh-positive}\\ \text{red cells}\end{matrix}\right\} + \left\{\begin{matrix}\text{Univalent Rh antibodies}\\ \text{(Blockers or glutinins)}\end{matrix}\right\} + \left\{\begin{matrix}\text{Conglutinin}\\ \text{(X protein)}\end{matrix}\right\} \rightarrow \text{Conglutination}$$

While X protein is heat stable, it has the peculiarity that it dissociates readily into its constituent smaller molecules of albumin and globulin upon slight dilution of the plasma with water. This explains why glutinins do not give rise to clumping under the conditions existing in the agglutination test, because the use of saline in preparing the red blood cell suspensions and for diluting the patient's serum is sufficient to dissociate partly or completely the X protein. The conglutination test is therefore carried out like the agglutination test except that the use of saline or any crystalline solution is strictly avoided. The red cells are suspended in the individual's own plasma or serum or more conveniently in inactivated Group AB serum. The patient's serum is tested undiluted, or in titrations the serum is diluted with Group AB serum instead of saline solution. I believe that the slide test of Diamond and Abelson,[34, 35] and the capillary tube test of Chown,[36] when properly carried out, depend for their success on a conglutination reaction rather than on an agglutination reaction, as originally stated by these workers. Since most Rh antisera will contain both univalent and bivalent antibodies, then, depending on the conditions of the test, one will obtain agglutination or conglutination or both.

In tests for Rh sensitization by the conglutination technique, positive reactions will be obtained in all cases in which the patient's serum contains Rh agglutinins or Rh blocking antibodies, or both, and occasionally when the other two tests are negative or inconclusive.[29, 35] The conglutination test is the most reliable indication of the presence or absence of Rh sensitization because it is always or almost always positive if the patient is sensitized. Moreover, glutinin is univalent and therefore presumably comprised of small molecules, so this antibody, like blocking antibody, plays a more important role than Rh agglutinin in the pathogenesis of congenital hemolytic disease.[19]

The bizarre behavior of congenital hemolytic disease, so that some infants do not present signs of hemolysis until several hours or days after birth, can probably be attributed to X protein, the third component necessary for in vitro conglutination, and therefore probably important for in vivo hemolysis.[29] Perhaps in these cases X protein does not form until after birth, at which time the profound physiologic changes occurring bring about aggregation of serum proteins into more complex molecules. In this connection Pedersen[37] has found

that serum from cow's fetus and newborn calves and foals contains large amounts of a special globulin of low molecular weight, designated fetuin by him, which he believes to be one of the precursors of X protein. Fetuin has also been found in human umbilical cord serum, although in smaller amount.

Rh BLOOD TYPES

Before proceeding with the presentation of illustrative clinical material, a brief description of the Rh blood types is in order.

While our antirhesus guinea pig and rabbit sera all gave parallel reactions,[10] agglutinating the blood of approximately 85 per cent of all white individuals, it was soon found that human antisera from Rh-negative patients sensitized to the Rh factor varied in their specificities. The great majority of the human antisera gave reactions identical with those of the antirhesus sera; such human antisera are now designated as standard anti-Rh or anti-Rh_0.[39] The variations among human antisera were soon explained by the demonstration of two additional varieties of Rh agglutinins, one giving 70 per cent positive reactions[18, 38] and designated[39, 40] as anti-Rh′, the other giving 30 per cent positive reactions[41, 42] and designated anti-Rh″. If blood specimens are tested with all three antisera, anti-Rh_0, anti-Rh′, and anti-Rh″, eight distinct types of human blood can be identified instead of the two, Rh positive and Rh negative, detected with the aid of standard human anti-Rh_0 serum or animal antirhesus serum alone. As I[43] have shown, the three Rh antisera detect three corresponding Rh factors, Rh_0, Rh′ and Rh″, which in combination give rise to at least five Rh agglutinogens instead of one, namely, Rh_0, Rh′, Rh″, Rh_1 (or Rh_0'), and Rh_2 (or Rh_0''). These five agglutinogens in combination determine the eight Rh types, which are hereditarily transmitted by means of at least six allelic genes,[44] *Rh_0, Rh′ Rh″, Rh_1, Rh_2,* and *rh.*

Anyone familiar with the serology and genetics of the four Landsteiner blood groups can readily master the eight Rh blood types. First, considering only the reactions of anti-Rh′ and anti-Rh″, these two antisera determine four types of blood entirely analogous serologically and genetically to the four blood groups. If, in addition, the reactions with anti-Rh_0 serum are taken into account, eight types of blood result, as shown in Table I. The types are named

TABLE I. THE EIGHT RH BLOOD TYPES*

BLOODS CONTAINING Rh_0				BLOODS LACKING Rh_0			
DESIGNATIONS OF TYPES	REACTIONS WITH SERA			DESIGNATIONS OF TYPES	REACTIONS WITH SERA		
	anti-Rh′	anti-Rh″	anti-Rh_0		anti-Rh′	anti-Rh″	anti-Rh_0
Rh_0	−	−	+	Rh−	−	−	−
Rh_1 (Rh_0')	+	−	+	Rh′	+	−	−
Rh_2 (Rh_0'')	−	+	+	Rh″	−	+	−
Rh_1Rh_2 ($Rh_0'Rh_0''$)	+	+	+	Rh′Rh″	+	+	−

*Wiener, A. S.: Am. J. Clin. Path. 15: 106, 1945.

after the antisera with which they react.[39, 40] For example, type Rh_0 reacts with anti-Rh_0 but not with anti-Rh′ or anti-Rh″; similarly, type Rh′ reacts with anti-Rh′ but not with anti-Rh_0 or anti-Rh″; etc. Blood reacting with anti-Rh′ and anti-Rh_0 but not with anti-Rh″ is designated Rh_1 (short for Rh_0') instead of Rh_0Rh′ because the factors Rh_0 and Rh′ in such blood are ordinarily hereditarily transmitted as a unit, like the agglutinogens A_1 and A in blood of Subgroup A_1 rather than like the agglutinogens A and B in Group AB blood. Thus, in the mating Rh negative × Rh_1, as a rule, either the children are all type Rh_1, or

one-half belong to type Rh_1 while the other one-half are Rh negative. To be sure, the factors Rh_o and Rh′ will segregate in occasional matings involving type Rh_1 individuals, namely, when type Rh_1 individual belongs to genotype Rh_oRh', but such cases are rare and have not yet been encountered because of the low frequencies of genes Rh_o and Rh'. For the same reason, blood reacting with anti-Rh″ and anti-Rh_o but not with anti-Rh′ is designated as type Rh_2 (or Rh_o″) rather than Rh_oRh″. On the other hand, types Rh′Rh″ and Rh_1Rh_2 are so designated because, with rare exceptions, such bloods contain two separate agglutinogens.

The Rh blood types, like the blood groups and M-N types, exhibit striking differences in distribution in various races[44] and promise to have important applications in anthropology. Some of the findings on the racial distribution of the Rh blood types are summarized in Table II. Chinese,[45, 46] Japanese,[47, 48]

TABLE II. DISTRIBUTION OF THE RH BLOOD TYPES

POPULATION TESTS	NUMBER OF PERSONS TESTED	FREQUENCIES OF Rh TYPES (PER CENT)							
		Rh–	Rh_1	Rh_2	Rh_1Rh_2	Rh_o	Rh′	Rh″	Rh′Rh″
Caucasians (New York City) a*	1000	12.9	54.1	12.8	16.4	2.6	0.9	0.3	0
b†	818	13.5	55.6	15.8	12.0	1.7	1.1	0.3	0
c‡	2438	14.5	52.5	15.7	13.1	2.4	1.1	0.7	0.02
Caucasians (England)§§§	927	14.8	54.9	12.2	13.7	2.5	0.7	1.3	0
Caucasians (Australia)§	350	14.9	54.0	12.6	16.5	0.6	0.9	0.5	0
Negroes (New York City)**	223	8.1	20.2	22.4	5.4	41.2	2.7	0	0
Asiatic Indians††	156	7.1	70.5	5.1	12.8	1.9	2.6	0	0
Chinese‡‡	132	1.5	60.6	3.0	34.1	0.8	0	0	0
Mexican Indians§§	98	0	48.0	9.2	41.8	1.0	0	0	0
Japanese a***	150	1.3	37.4	13.3	47.3	0	0	0	0.7
Japanese b†††	180	0.6	51.7	8.3	39.4	0	0	0	0
Filipinos§	100	0	87.0	2.0	11.0	0	0	0	0
Indonesians§	100	0	75	2	22	0	0	0	1

*Old Series; Wiener, A. S.: Am. J. Clin. Path. 15: 106, 1945.
†New Series; Wiener, A. S., and Sonn, E. B.: Bull. New York Acad. Sc. In press.
‡Unger, L. J.: Personal Communication.
§Simmons, R. T.: Personal Communication.
**Wiener, Belkin, and Sonn.[53]
††Wiener, Sonn, and Belkin: J. Immunol. 50: 341, 1945.
‡‡Wiener, Sonn, and Yi.[46]
§§Wiener, Zepeda, Sonn, and Polivka.[50]
***Waller and Levine.[47]
†††Miller and Taguchi.[48]
§§§Race, R. R.: Personal communication.

American Indians,[49, 50] Filipinos, Hawaiians,[51] and Australian aborigines[52] are characterized by the virtual absence of the Rh-negative type; in Negroes[53, 57] the most striking finding is the high incidence of type Rh_o, which is about twenty times as frequent as in the white race. Some correlation between the frequency of the Rh-negative type in the population and the incidence of congenital hemolytic disease is to be expected, and it is of interest to note that Levine and Wong[45] report that this disease is virtually unknown among Chinese.

The theory of six allelic genes has been tested by studying the Rh blood types in families[54-56] and by the gene frequency method; the findings support the theory in its essential details. In Table III are presented our findings on the heredity of the Rh blood types in 197 families with 463 children. Only two apparent contradictions to the genetic theory were encountered and both of these proved to be due to illegitimacy. In the statistical test by the gene frequency method, the findings on white individuals[57, 58] have been in good agreement with the theory of six allelic genes. In American Indians and Mongolians, however,

TABLE III. SUMMARY OF AUTHOR'S FAMILY STUDIES ON THE RH BLOOD TYPES*

MATING	NUMBER OF FAMILIES	NUMBER OF CHILDREN OF TYPES							
		Rh–	Rh_1	Rh_2	Rh_1Rh_2	Rh_o	Rh′	Rh″	TOTALS
Neg. × Neg.	4	14	0	0	0	0	0	0	14
Neg. × Rh_1	49	25	73	0	0	7	0	0	105
Neg. × Rh_2	16	10	0	20	0	0	0	0	30
Neg. × Rh_1Rh_2	15	(1)	18	14	0	0	0	0	33
Neg. × Rh_o	3	3	0	0	0	3	0	0	6
Neg. × Rh′	2	1	0	(1)	0	0	1	0	3
Neg. × Rh″	1	1	0	0	0	0	0	5	6
Rh_1 × Rh_1	26	5	62	0	0	4	1	0	72
Rh_1 × Rh_2	21	6	15	7	18	0	2	0	48
Rh_1 × Rh_1Rh_2	27	0	46	8	25	0	0	0	79
Rh_1 × Rh_o	1	1	0	0	0	0	0	0	1
Rh_1 × Rh′	3	0	3	0	0	0	0	0	3
Rh_1 × Rh″	4	1	2	0	3	0	0	0	6
Rh_2 × Rh_2	2	1	0	4	0	0	0	0	5
Rh_2 × Rh_1Rh_2	6	0	2	8	8	0	0	0	18
Rh_2 × Rh_o	2	0	0	1	0	4	0	0	5
Rh_1Rh_2 × Rh_1Rh_2	10	0	3	1	17	0	0	0	21
Rh_1Rh_2 × Rh_o	2	0	1	1	0	0	0	0	2
Rh_1Rh_2 × Rh′	2	0	1	1	2	0	0	0	4
Rh_o × Rh′	1	0	0	0	0	1	1	0	2
Totals	197	69	226	66	73	19	5	5	463

*Wiener and Sonn.[56]

the calculated sum of the gene frequencies falls short of 100 per cent, and this we[50] attribute to the occurrence in these peoples of an exceptionally high frequency of the so-called genes Rh_y and/or Rh_z of Race and Taylor.[59, 60] Negroes are characterized by an exceptionally high percentage of individuals with blood giving weak or intermediate reactions with one or more of the Rh antisera,[57] and these have been attributed to the existence of still more genes in the Rh allelic genes,[61] the "intermediate genes." For practical purposes, the theory of six allelic genes has proved adequate, because the genes Rh_y and/or Rh_z and the intermediate genes are quite rare among white individuals. Under the theory of six allelic genes, twenty-one genotypes are possible, and the genotypes corresponding to each of the eight Rh blood types are readily ascertained, as shown in Table IV. If the genes Rh_y and/or Rh_z and the intermediate genes are taken into account, the situation naturally becomes far more complicated.

TABLE IV. THE EIGHT RH TYPES AND THEIR GENOTYPES

Rh TYPES	GENOTYPES
Neg.	*rh′rh*
Rh′	*Rh′Rh′* and *Rh′rh*
Rh″	*Rh″Rh″* and *Rh″rh*
Rh′Rh″	*Rh′Rh″*
Rh_o	Rh_oRh_o and Rh_orh
Rh_1(Rh_o′)	Rh_1Rh_1, Rh_1rh, Rh_1Rh', Rh_1Rh_o, and $Rh'Rh_o$
Rh_2(Rh_o″)	Rh_2Rh_2, Rh_2rh, Rh_2Rh'', Rh_2Rh_o, and $Rh''Rh_o$
Rh_1Rh_2(Rh_o′Rh_o″)	Rh_1Rh_2, Rh_1Rh'', and $Rh'Rh_2$

THE HR FACTOR

The literature on the Hr factor is somewhat confused, because the early work was done with weak antisera, so that some of the findings were inaccurate and misleading. Levine and Javert[62] first reported that they had detected in the serum of an Rh-positive mother of an erythroblastotic infant an antibody which had the property of agglutinating all Rh-negative bloods. Because of this property of the serum, the new agglutinable property detected by it was designated as Hr and the corresponding agglutinin as anti-Hr. Levine reported that his anti-Hr serum gave about 30 per cent positive reactions and that all Rh-

positive bloods not agglutinated by anti-Rh′ reacted with anti-Hr. On this basis he postulated that Hr and Rh were allelic, without further clarifying his hypothesis. He also asserted that Hr incompatibility must be considered as a possibility whenever the Rh-positive mother has an Rh-negative child[63] and that all type Rh_1Rh_2 individuals are Hr negative.[47] As will be shown, the latter two statements have been contradicted by subsequent findings.

In 1943, Race and Taylor[64] also detected in the serum of an Rh-positive mother of an erythroblastotic infant an agglutinin which reacted with all Rh-negative bloods but differed from Levine and Javert's findings in that it gave 80 per cent positive reactions. As has been pointed out by Wiener and coworkers,[65] however, Race and Taylor's serum detected the same blood property as Levine and Javert's serum, the lower percentage of positive reactions obtained by Levine being due to the use of a weaker antiserum which missed bloods heterozygous for the Hr factor. In this lecture, therefore, the blood property studied by the British investigators will also be designated as Hr. As postulated by Race and associates,[66] Hr is a blood factor present in the agglutinogens determined by genes *Rh₂*, *Rh″*, *Rh₀*, and *rh* but absent from the agglutinogens determined by genes *Rh₁* and *Rh′*. From the reactions determined by the six genes (Table V), the reactions, of the twenty-one genotypes are readily ascer-

TABLE V. THE REACTIONS DETERMINED BY THE SIX STANDARD GENES AND GENES *Rhy* AND *Rhz*

GENES	REACTIONS WITH Rh SERA			REACTIONS WITH Hr ANTISERUM
	anti-Rh′	anti-Rh″	anti-Rh_0	
rh	Neg.	Neg.	Neg.	Pos.
Rh_0	Neg.	Neg.	Pos.	Pos.
Rh′	Pos.	Neg.	Neg.	Neg.
Rh_1	Pos.	Neg.	Pos.	Neg.
Rh″	Neg.	Pos.	Neg.	Pos.
Rh_2	Neg.	Pos.	Pos.	Pos.
Rh_y	Pos.	Pos.	Neg.	Neg.
Rh_z	Pos.	Pos.	Pos.	Neg.

tained, as shown in Table VI. In giving the expected reactions with anti-Hr serum, it is postulated that a single dose of a gene positive for Hr determines a weaker reaction with anti-Hr than a double dose of Hr-positive genes. (This idea is supported by observations made in tests with weak anti-Hr serum,[64, 65] while in tests with potent Hr antisera it is difficult or impossible to demonstrate the gene-dose effect.) It will be seen that the expected percentage of positive reactions, approximately 80 per cent, closely agree with that reported by Race and Taylor and that observed by me. If only the strong reactions are counted, then the percentage of positives becomes 30 per cent, as reported by Levine. This explains the apparent contradiction between the report of Levine, and Race and Taylor, and why Levine thought that type Rh_1Rh_2 bloods were Hr negative. Since Hr-negative mothers can only belong to genotype *Rh_1Rh_1*, *$Rh_1Rh′$*, or *Rh′Rh′*, they must transmit either an *Rh_1* or an *Rh′* gene to every child and so cannot have any Rh-negative children, contrary to the assertion made by Levine.

The main clinical application of the Hr factor is as a presumptive test for homo- or heterozygosity of type Rh_1 fathers in families with erythroblastotic infants.[64] The diagnosis is only *presumptive* because there are five genotypes in type Rh_1, while anti-Hr serum merely subdivides type Rh_1 into two parts. Hr-negative individuals of type Rh_1 are almost surely homozygous for *Rh_1*, while

TABLE VI. RELATION OF THE HR FACTOR TO THE RH BLOOD TYPES

Rh BLOOD TYPE	REACTIONS WITH Rh SERA			GENOTYPES	REACTIONS WITH Hr ANTISERUM*	PER CENT OBSERVED (350 TESTS ON WHITE INDIVIDUALS, N.Y.C.)
	anti-Rh′	anti-Rh″	anti-Rh_0			
Rh-	Neg.	Neg.	Neg.	*rhrh*	Strong	12.3
Rh′	Pos.	Neg.	Neg.	*Rh′Rh′*	Neg.	0
				Rh′rh	Weak	1.1
Rh″	Neg.	Pos.	Neg.	*Rh″Rh″*, *Rh″rh*	Strong	0.9
Rh′Rh″‡	Pos.	Pos.	Neg.	*Rh′Rh″*	Weak	0
Rh_0	Neg.	Neg.	Pos.	Rh_0Rh_0, Rh_0rh	Strong	1.1
Rh_1	Pos.	Neg.	Pos.	Rh_1Rh_1, Rh_1Rh'	Neg.	19.7
				Rh_1rh, Rh_1Rh_0, $Rh'Rh_0$	Weak	36.9
Rh_2	Neg.	Pos.	Pos.	Rh_2Rh_2, Rh_2Rh'', Rh_2rh, Rh_2Rh_0, $Rh''Rh_0$	Strong	16.0†
Rh_1Rh_2‡	Pos.	Pos.	Pos.	Rh_1Rh_2, Rh_1Rh'', $Rh'Rh_2$	Weak	11.7
				Rh_1Rh_2	Neg.	0.3

*Theoretically expected reactions. Actually the Hr antiserum used was of such high titer that all positive bloods gave uniformly strong reactions.

†Includes one blood giving intermediate reactions (Rh_2^1).

‡If the existence of both genes Rh_Y and Rh_z is admitted, then there are nineteen genotypes possible in these types instead of four as given in Table IV. The additional fifteen genotypes, if they actually exist, must all be quite rare, so for simplicity only the most common, genotype Rh_1Rh_z, is given in this table.

Hr-positive individuals of type Rh_1 are almost always heterozygous and bear gene *rh*.

The accuracy of the theory of Race and co-workers regarding the Hr factor has been confirmed by family studies and by the gene frequency method.[65, 67] Since every gene negative for factor Rh′ is positive for factor Hr, it is evident that factors Rh′ and Hr are related to each other serologically and genetically like M and N (see Wiener and co-workers,[65] Levine[69]). To indicate this (especially since Fisher[70] has postulated the existence of three factors in Rh-negative blood corresponding to the three Rh factors), the standard Hr factor which we have described is perhaps better known as Hr′ and the corresponding serum as anti-Hr′. Of the other two Hr factors postulated by Fisher, Hr″ has recently been found by Mourant,[71] while convincing evidence is still lacking concerning the existence of Hr_0.[68]

CLINICAL APPLICATION

A few cases will now be described which illustrate the application of the Rh blood types in relation to diagnosis, treatment, and prognosis of congenital hemolytic disease.

CASE 1.—I was requested by Dr. J. Hoffman, at the Israel Zion Hospital, to see a deeply jaundiced infant, 2 months of age, in order to determine whether he was suffering from congenital stricture of bile ducts or congenital hemolytic disease.

The mother of the infant gave the following obstetric history: The first pregnancy terminated at term, in 1935, with a normal female child. The second pregnancy, in 1939,

yielded a male infant who developed anemia after birth but recovered following transfusion therapy. The third pregnancy, in 1945, resulted in the patient, who shortly after birth developed jaundice and anemia for which he was treated by transfusions of Rh-negative blood. The anemia improved, but deep jaundice persisted.

The infant's mother had never received a blood transfusion.

The grouping and Rh tests gave the results listed in Table VII.

The fact that the mother was Rh negative, while the father and all three children were Rh positive could, of course, be merely coincidental. However, in the maternal serum anti-Rh_0 agglutinins of titer 16 were detected, demonstrating that she was sensitized to the Rh factor and establishing the diagnosis of congenital hemolytic disease.

TABLE VII

BLOOD OF	GROUPS AND SUBGROUPS	MN TYPE	Rh TYPE	Hr REACTION
Father	O	M	Rh_2	Pos.
Mother	A_2	MN	Neg.	Pos.
Ten-year-old sister	O	MN	Rh_2	Pos.
Six-year-old brother	A_2	MN	Rh_0	Pos.
Patient	O	MN	Rh_2	Pos.

The case is typical in that it demonstrates how the first Rh-positive infant escapes the disease, while the subsequent infants are affected progressively more severely as the sensitization of the mother increases in degree with each pregnancy. The second infant recovered fully even although the donors whose blood was used for the transfusions were not selected with regard to the Rh factor, then unknown. The patient was treated properly with transfusions of Rh-negative blood and nevertheless had an unusual complication in that damage to the liver finally caused death at the age of 3 months.

The Hr factor is of no assistance in determining hetero- or homozygosity of the father in this case, because he does not belong to type Rh_1 but to type Rh_2. The fact that he has had children of types Rh_2 and Rh_0 proves that he belongs to genotype Rh_2Rh_0. Therefore, every future child of this couple must be Rh positive, either type Rh_2 or Rh_0, and would be certain to have congenital hemolytic disease.

CASE 2.—This patient was referred by Dr. B. Merriam with the history that she was in the eighth month of pregnancy and that the fetal heart sounds had not been audible for the past three weeks. This was the patient's third pregnancy. The first pregnancy had terminated with a normal female child 4 years old at this time, while the second pregnancy had yielded a seven-month macerated fetus.

Tests on the patient, her husband, and daughter showed the results listed in Table VIII.

TABLE VIII

BLOOD OF	GROUP AND SUBGROUP	MN TYPE	Rh TYPE	Hr REACTION
Husband	O	MN	Rh_1	Neg.
Patient	A_2	M	Neg.	Pos.
Daughter	A_2	M	Rh_1	Pos.

No Rh agglutinins were demonstrable in the patient's serum, but Rh blocking antibodies were present in low titer (2 units). By the conglutination technique, Rh antibodies were readily demonstrable in the patient's serum in dilutions as high as 1 to 50. These findings therefore established the diagnosis of congenital hemolytic disease as the cause of the stillbirth after the second pregnancy and the intrauterine death during the present pregnancy.

Shortly after these tests were carried out the patient gave birth to a macerated fetus, after which she had a hemorrhage which was treated by plasma transfusions. The patient probably owes her life to the newer knowledge of the Rh blood factors and the blocking antibodies. Under similar circumstances before the discovery of the Rh factor, the patient

would have been treated by transfusions of whole blood from an unselected and therefore probably Rh-positive, Group A donor, especially since blocking antibodies are not detectable in the usual cross-matching tests.

The more severe manifestations of congenital hemolytic disease in this case, as contrasted with Case 1, is in line with the remarks concerning the more serious prognostic significance of Rh blocking antibodies as compared with Rh agglutinins. Moreover, the husband of the patient is Hr negative so that the prognosis for further pregnancies for this couple is practically holpeless, because every future pregnancy would be expected to yield an Rh-positive fetus and therefore terminate with another stillbirth. In Case 1, on the other hand, where the degree of Rh sensitization of the mother is not as pronounced, future children, although certain to be affected with congenital hemolytic disease, might nevertheless prove viable and recover after transfusion therapy.

CASE 3.—This case was referred to me by Dr. I. Frohman, who is reporting it in detail elsewhere.[72] The patient was first admitted to the Rockaway Beach Hospital during the sixth month of her eleventh pregnancy because of an abruptio placentae. She was delivered shortly afterward of a stillborn fetus with hydrops.

The obstetric history and the results of grouping and Rh tests are given in Tables IX and X.

No anti-Rh agglutinins were demonstrable in the patient's serum, but Rh blocking antibodies were present in low titer (2 units). These findings therefore indicate that the stillbirths that this patient had after the fifth pregnancy were due to congenital hemolytic disease. The stillbirth occurring after the third pregnancy must be attributed to some mechanism other than isoimmunization in pregnancy.

TABLE IX

BLOOD OF	GROUP	MN TYPE	Rh TYPE
Husband	O	MN	Rh_2
Patients	O	N	Neg.

TABLE X

PREGNANCIES	GROUP	MN TYPE	Rh TYPE
1. 1928—Normal male infant	O	MN	Rh_2
2. 1929—Normal male infant	O	MN	Rh_2
3. 1930—Premature stillbirth at 6½ months; Patient had hypertension during pregnancy			
4. 1932—Normal male infant	O	N	Neg.
5. 1935—Female infant; jaundice on eighth day; cleared in a few days	O	N	Rh_2
6. 1936—Normal male infant	O	MN	Neg.
7. 1938—Stillborn male			
8. 1938—Fraternal twins in separate sacs; male twin normal; female twin died after thirteen hours from icterus gravis and hydrops	O	MN	Neg.
9. 1940—Normal male infant	O	N	Neg.
10. 1943—Normal male infant	O	N	Neg.
11. 1944—Stillbirth (present pregnancy)			

The husband of this patient, in contrast to the fathers in Cases 1 and 2, is heterozygous for the Rh factor and belongs to genotype Rh_2rh. It would therefore be expected that one-half of the children of this couple should be Rh positive (type Rh_2), and one-half Rh negative. If the stillbirths are counted as Rh positive, a close approximation is obtained to the expected 1:1 ratio. In future pregnancies, this couple has a 50 per cent chance of having an Rh-negative and therefore normal child. Should such a fetus be Rh positive, it almost surely would be stillborn.

In this case it took more than one pregnancy with Rh-positive fetuses to sensitize the mother sufficiently to give rise to infants with hemolytic disease.

Nevertheless, the sensitization, when it finally did develop, was intense, as evidenced by the presence of blocking antibodies in the maternal serum and the occurrence of stillbirths in the seventh, eighth, and eleventh pregnancies. The mild jaundice exhibited by the female infant born after the fifth pregnancy was probably a manifestation of a very mild hemolytic disease. After the fifth pregnancy the sensitization became more pronounced so that only Rh-negative infants survived.

Of particular interest is the eighth pregnancy which yielded a pair of unlike-sexed twins. The male twin was Rh negative and normal, while the female twin died within thirteen hours from icterus gravis, undoubtedly because she was Rh positive.

CASE 4.—The patient was a male infant, 6 days of age, from the service of Dr. David A. Meyers at the Adelphi Hospital. The infant was the eighth born to his parents. The first seven children had been all normal at birth, and all were alive except the oldest, who had died as a result of an automobile accident. On the second day of life the patient was noticed to be jaundiced. The jaundice deepened and by the fifth day the hemoglobin concentration had dropped to 63 per cent, the red cell count was 2.9 millions, and the blood film showed 5 nomoblasts per 100 white blood cells.

Blood tests on the parents and children showed the results given in Table XI.

TABLE XI

BLOOD OF	GROUP	MN TYPE	Rh TYPE	HR REACTION
Father	O	M	Rh_1	Neg.
Mother	O	M	Neg.	Pos.
1. Brother born 1931 (not alive)				
2. Sister born 1933	O	M	Rh_1	Pos.
3. Sister born 1938	O	M	Rh_1	Pos.
4. Sister born 1939	O	M	Rh_1	Pos.
5. Sister born 1941	O	M	Rh_1	Pos.
6. Brother born 1942	O	M	Rh_1	Pos.
7. Sister born 1943	O	M	Rh_1	Pos.
8. Patient	O	M	Rh_1	Pos.

The maternal serum contained no detectable Rh agglutinins and gave only a questionable blocking reaction, but by the conglutination method, Rh antibodies of titer 16 were readily demonstrable, proving that the patient's mother was sensitized to the Rh factor and establishing the diagnosis of congenital hemolytic disease. By the conglutination technique Rh antibodies were also readily detected in the infant's plasma, and autoagglutination of the infant's red cells by his own plasma was demonstrable. This corroborates the remarks concerning the ease with which maternal univalent antibodies pass through the placental barrier into the fetal circulation.

A remarkable feature of this case is the large number of pregnancies required to induce Rh sensitization, despite the fact that every child was Rh positive. There is no doubt that the father is homozygous (genotype Rh_1Rh_1) because he is Hr negative and every one of the seven children tested belongs to type Rh_1. If this couple has any more children, such children are certain to have congenital hemolytic disease.

For treating the infant, 200 c.c. of blood were obtained from a Group 0, Rh-negative professional donor. One-half of the blood was transfused at once and the remainder on the following day. The infant improved immediately, the jaundice cleared, and when the patient was seen a week later, the hemoglobin was 85 per cent. He was discharged from the hospital, and the parents were instructed to return with him in a week. He was not brought back until he was five weeks old, however, and by that time the hemoglobin had dropped to 40 per cent. Two hundred cubic centimeters of blood were then drawn from the patient's mother and the washed and packed red cells (about 80 c.c.) transfused by syringe and two-way stopcock into a scalp vein. The hemoglobin rise this time was sustained, and complete recovery followed.

The presence of Rh antibodies in the infant's serum, as demonstrated in this and other cases, is the rationale for the use of Rh-negative blood when treating infants with hemolytic disease, even though the infants themselves are Rh posi-

tive. When Rh-negative donors are not available, it is convenient to transfuse the mother's washed cells, and if the packed red cells are transfused, it is possible to get the effect of a transfusion of twice the volume of whole blood. The use of maternal washed cells is permissible even though the mother's blood belongs to an incompatible blood group, and preliminary grouping and Rh and cross-matching tests may be dispensed with if facilities for carrying out the tests are not available.

CASE 5.—A physician in Santiago, Chile, reported that his wife was in the sixth month of her third pregnancy and showed signs of hydramnios. He was concerned because he had found his wife to be Rh negative and himself Rh positive. He could not detect any Rh agglutinins in his wife's serum, but the patient gave the following significant obstetric history:

The first pregnancy occurred in 1943. About three months after marriage the patient had a ruptured ectopic gestation for which an operation was performed. After the operation she was given two transfusions of Group A bank blood, not selected for the Rh factor. There were no untoward reactions following these transfusions.

The second pregnancy occurred in 1944 and terminated with an eight-month macerated fetus.

Grouping and Rh tests were carried out on blood samples mailed from Chile, with the results given in Table XII.

No Rh agglutinins could be detected in the patient's serum, but blocking antibodies were present in high titer (50 units), and by the conglutination technique, Rh antibodies of titer 90 were demonstrable. In view of the high titer of Rh blocking antibodies in the patient's serum, and the fact that her husband was Hr negative, the prognosis for the present pregnancy was grave. In fact, the pregnancy soon did terminate with a seven-month stillbirth.

TABLE XII

BLOOD OF	GROUP AND SUBGROUP	MN TYPES	Rh TYPE	Hr REACTION
Husband	O	MN	Rh_1	Neg.
Patient	A_2	MN	Neg.	Pos.

There is no doubt that the two blood transfusions given to this patient were the cause of her sensitization to the Rh factor, because the ectopic pregnancy had not advanced far enough to bring about Rh sensitization. Had Rh-negative blood been transfused, most likely the second pregnancy would have yielded a viable infant. This case serves to illustrate the importance of Rh tests before giving blood transfusions to women in the child-bearing age, as already pointed out.

CASE 6.—The patient was an infant who became pale and then jaundiced shortly after birth, so that blood transfusion therapy was necessary. The infant was his mother's second child; the first pregnancy had terminated with a normal female child 4 years of age at this time.

Grouping tests on the infant, his parents, and sister gave the result listed in Table XIII.

These results came as a surprise, because we had felt reasonably sure from the clinical picture that the infant had congenital hemolytic disease. However, this diagnosis could now be excluded, especially since the maternal sera contained no abnormal isoantibodies.

TABLE XIII

BLOOD OF	GROUP	MN TYPE	Rh TYPE	Hr REACTION
Father	O	MN	Rh_2	Pos.
Mother	O	M	Rh_1Rh_2	Pos.
Sister (4 years of age)	O	MN	Rh_2	Pos.
Patient	O	M	Rh_1	Pos.

The history was then obtained that the patient had had a traumatic delivery. He soon died, despite transfusion therapy, and an autopsy revealed the presence of a ruptured liver with hemorrhage into the parenchyma of the liver.

CASE 7.—This case was referred by Dr. A. M. Young, of the Mount Sinai Hospital in Cleveland, Ohio, because the patient, a newborn infant, had all the clinical signs and symptoms of congenital hemolytic disease, yet mother and infant were both Rh positive. In the maternal serum an irregular agglutinin was detected, which I was requested to identify.

Grouping and Rh tests showed the results given in Table XIV.

Thus, the infant's red blood cells contain all three Rh factors, Rh_0, Rh′, and Rh″, while the maternal red cells contain only factors Rh_0 and Rh′. The presence in the maternal serum of an atypical immune isoagglutinin of titer 32 was corroborated. As expected, this proved to correspond in specificity to anti-Rh″.

TABLE XIV

BLOOD OF	GROUP AND SUBGROUP	MN TYPE	Rh TYPE
Mother	A_1	MN	Rh_1
Infant	A_1	MN	Rh_1Rh_2

While type Rh_1 mothers with type Rh_2 or type Rh_1Rh_2 infants and type Rh_2 mothers with type Rh_1 or type Rh_1Rh_2 infants are quite common, these combinations only rarely give rise to cases of congenital hemolytic disease, because factors Rh′ and Rh″ are much weaker antigens than Rh_0. As already mentioned, about 90 per cent of the clinical cases of congenital hemolytic disease can be traced to the Rh_0 factor.

CASE 8.[73]—An infant 12 days old was seen in consultation with Dr. Benjamin Bloom, because of the sudden appearance of jaundice and pallor the day before. The patient was the first child. Her mother had never had a previous pregnancy and had never received a blood or plasma transfusion. The patient was born at term but her birth weight was only 4 pounds, 11 ounces. In all other respects she appeared normal and was discharged from the hospital on the eighth day. At home, she made satisfactory progress until jaundice and anemia appeared on the eleventh day. Blood count performed on the twelfth day showed: hemoglobin, 50 per cent; red blood cells, 1.75 million per cubic millimeter; white blood cells, 17,000; polymorphonuclears, 41 per cent; lymphocytes, 53 per cent; monocytes, 6 per cent; no nucleated red cells on smear.

Grouping and Rh test showed the results given in Table XV.

Since the patient's mother belonged to type Rh_1Rh_2 and was Hr positive, this excluded the Rh factors and the Hr factor as having any bearing on the problem. The incompatibility in blood groups suggested that this might have some bearing on the problem, so the isoagglutinin titers of the maternal serum were determined. By the agglutination method the titers were about twenty-five times as high as the average normal titer, namely, the anti-A titer (for A_2 test cells) was 500, while the anti-B titer was 250. By the conglutination technique the titers were even higher, namely, 5,000 for anti-A and 500 for anti-B. In view of these findings, there could be no doubt but that this patient's hemolytic anemia was due to isoimmunization of the mother against the agglutinogen A in the infant's blood.

TABLE XV

BLOOD OF	GROUP AND SUBGROUP	MN TYPE	Rh TYPE	Hr REACTION
Father	A_1	N	Rh_0	Pos.
Mother	O	MN	Rh_1Rh_2	Pos.
Patient	A_1	N	Rh_1	Pos.

Two of the questions raised by this case are how congenital hemolytic disease developed in a firstborn in the absence of previous sensitization of the mother by transfusion and why the onset of the disease was delayed until the eleventh day. Probably the high anti-A titer found in the tests did not accurately reflect the titer during pregnancy because, for reasons already pointed out, the isoantibody titer rises after delivery. Since the infant had been breast fed, she probably ingested larger and larger quantities of anti-A glutinins as the maternal isoantibody titer rose, and finally enough antibody was absorbed by the infant to bring about hemolysis. This serves to emphasize the

importance of interdicting breast feeding in all cases of congenital hemolytic disease. Another factor possibly delaying hemolysis was the infant's immaturity judging from its low weight, and the corresponding immaturity of its X protein. In another milder case of congenital hemolytic disease in a firstborn infant due to A-B isoimmunization, the mother gave the significant history that two years previously she had received two transfusions of reconstituted, desiccated pooled plasma. Experiments performed by me demonstrate that the intravenous injection of as little as 40 c.c. of such plasma can induce a pronounced rise in titer of the isoagglutinins anti-A and anti-B. This suggests that plasma transfusions should not be given indiscriminately to women in the childbearing age, and it might also be mentioned that injections of therapeutic horse antisera may produce the same effect because such sera also contain A and B group substances.*

Tests on the infant's saliva showed her to be a secretor, thus disproving the contention that only nonsecretors are subject to congenital hemolytic disease due to A-B isoimmunization.

The infant was given a transfusion of 60 c.c. of the packed, washed red cells of her mother. When the patient was seen three days later, her hemoglobin had risen to 66 per cent. By the end of the week, however, the hemoglobin had fallen to 45 per cent, so a second transfusion of washed maternal red cells was given. Again the patient improved, and this time recovery was sustained.

CONCLUSIONS

With the laboratory methods now available, it is possible to diagnose accurately practically every case of congenital hemolytic disease or to exclude this diagnosis. It is also possible to specify more exactly the pathogenesis, whether due to isoimmunization to one or more of the Rh factors, Rh_0, Rh′ and Rh″, or to the Hr factors or the A-B-O factors.

The great majority of erythroblastotic infants born alive can be saved by transfusions of Rh-negative blood or washed maternal red cells. However, no effective method is known for saving infants from the lethal effects of certain hepatic injuries sustained as a result of the disease or from kernicterus or hemorrhagic complications. What underlies the constitutional predisposition which makes one individual easier to sensitize to the Rh factor than another is not clearly understood.

Future work should be concerned primarily with the prophylaxis and treatment of congenital hemolytic disease. This problem will not be completely conquered until effective methods have been devised for treating sensitized Rh-negative women so that they are capable of bearing normal Rh-positive infants.

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LABORATORY METHODS

STOMACH-TUBE FEEDING OF SMALL LABORATORY ANIMALS

A Simple "Hairpin" Gag Insuring Safe and Rapid Intubations by a Single Person

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ACCURATE oral administration of measured amounts of a drug to laboratory animals is best achieved with the help of a stomach tube. As early as 1908 Marks[1] described the use of a silk rubber catheter for the stomach-tube feeding of mice. His procedure was soon widely adopted and at present is almost universally used. Most investigators substituted a simple metal cannula (made from an intramuscular injection needle) for the silk rubber tube. This eliminated the necessity of a mouth gag, since, unlike a rubber tube, the needle could not be punctured or severed by the animal's teeth during the process of insertion and withdrawal. Recently, Ferril[2] proposed a similar metal cannula, made from an injection needle by soldering a brass knob to its end, for the feeding of rats. However, while Mark's procedure works well in mice and even small rats, the less experienced operator will encounter difficulties in feeding full-grown and untrained rats without fixation of the jaws and without any other immobilization.

The procedure generally adopted today in most research laboratories makes use of a wooden mouth gag with a central hole for the gum elastic catheter.[3, 4] The main drawback of this instrument is that it completely obscures the structures of the mouth and pharynx, so that the stomach tube has to be guided by touch rather than by sight. Thus difficulty may be encountered in attempts to pass the stomach tube above the animal's tongue. To overcome this obstacle, the tongue has to be forced and held forward between the gag and the animal's lower jaw. This requires that the gag be "put well back into the rat's mouth at the moment of insertion"[3] and usually also be rotated around its long axis counter clockwise when held with the right hand. This process is time consuming and, because of the crude shape of wooden mouth gags, is likely to hurt the animal and to stimulate violent struggling, which in turn may result in abrasion wounds of the gums. Obviously this is particularly undesirable if repeated intubations have to be carried out.

To obviate these difficulties, a simple instrument was devised which has proved its merits in more than six years of continuous use in many thousands of stomach-tube feedings of albino rats performed by my associates and me.[5] It consists of a piece of strong wire shaped in the form of a hairpin, the ends of which rest in a short metal bar. A metal rod, attached to the opposite side of this bar, permits fixation of the "hairpin" gag to a standard laboratory support. The dimensions and proportions of the instrument are evident from Fig. 1, which also illustrates stomach tubes of various sizes and makes. The

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gadget can be easily improvised for temporary use by forcing the branches of a strong hairpin into a cork stopper, which rests in a double jawed clamp. Without fixation and with appropriate increase in size, the instrument will serve with advantage for larger laboratory animals.

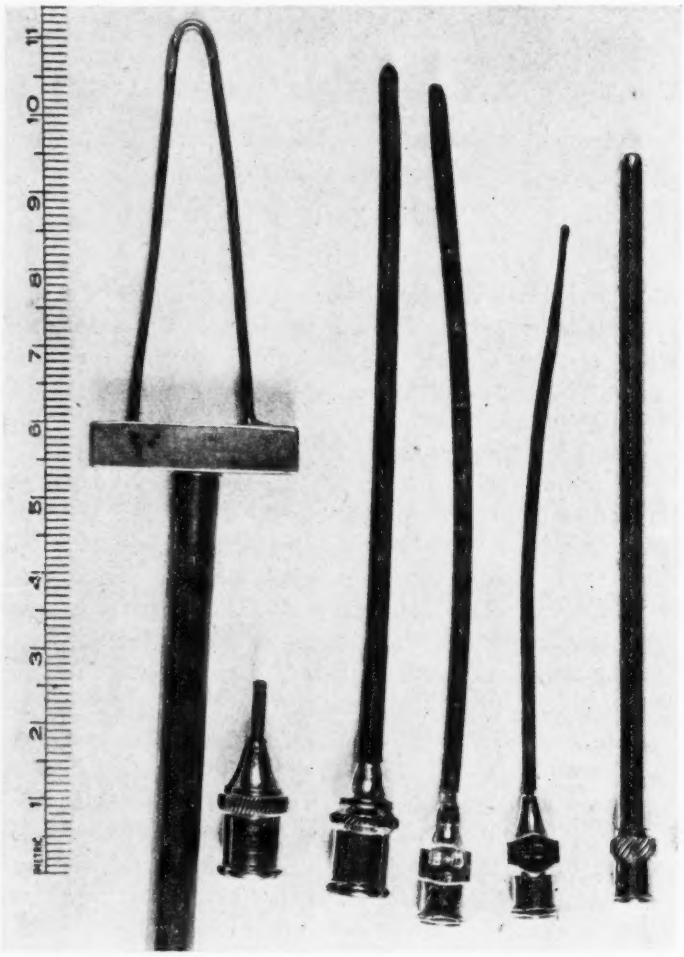

Fig. 1.—From left to right: "hairpin" gag, metal syringe adapter, soft rubber tube, silk rubber tube, silk rubber tube for small rats, metal cannula.

Before intubation, the rat is easily and quickly immobilized by wrapping it comfortably in a small towel (size of a large napkin, folded once) in such a way that the escape of the front limbs is prevented, whereas the head and neck remain free. The wrapping is best accomplished in the following manner: the folded towel, with the folding line pointing cranially, is placed over the animal's back not including the head; the rat is then grasped through the towel with the left hand and lifted into the air, while the right hand guides the left free end of the towel ventrally over the front legs, thus forming a "funnel" with its narrowest circumference around the animal's neck. Fixation of the towel at the neck is achieved with the help of a large artery clamp which is gently closed over the towel so as to include a part of the fur between its ends. This manipulation takes only a few seconds in actual performance.

The rat is now grasped firmly at the back of its head just behind the ears with the thumb and forefinger of the left hand, while the back of the middle finger presses against its vertebral column, thus enforcing extension of the

head and slight opening of the mouth. This grip completely immobilizes the animal. The towel, hanging like a cloak around its body, prevents scratching or violent movements. Under slight upward pressure maintained by the left hand throughout the intubation, the jaws of the rat can now be readily slid on sideways over the narrow end of the "hairpin."

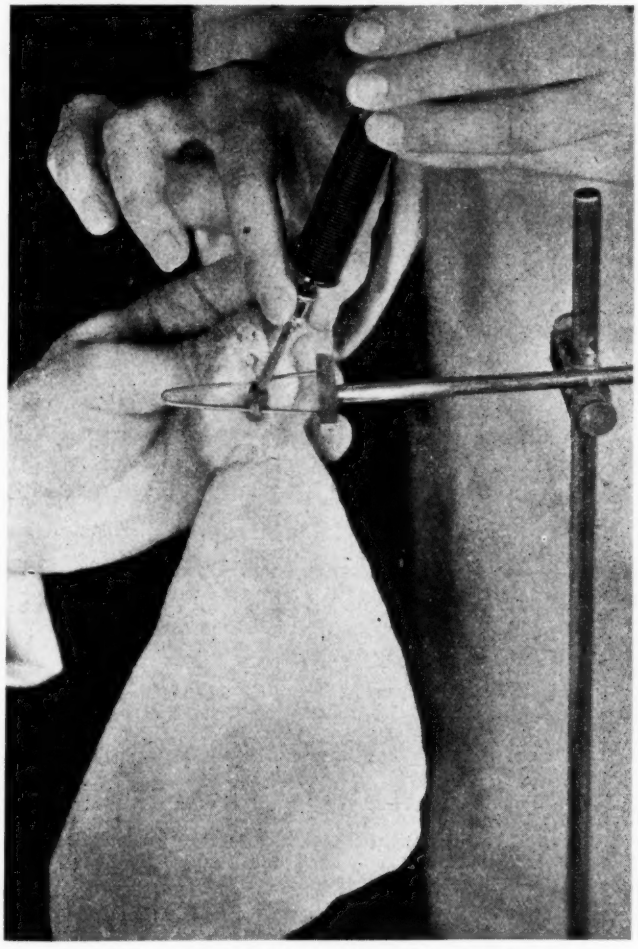

Fig. 2.—Stomach-tube feeding with assistance.

As is apparent from Fig. 2, fixation of the gag to the laboratory stand is carried out in such a way that the plane determined by the triangular device forms an angle of about 45 degrees with the horizontal. The wedgelike shape of the instrument allows good adjustment to the size of the animal's mouth. With the rat in feeding position, all structures of the mouth are exposed and clearly visible.

The syringe, containing the desired amount of drug, and the attached stomach tube are now grasped with the operator's right hand and without using force the tube is gently guided downward above the animal's tongue (Fig. 2) until its length is lost in the digestive tract and mouth; the plunger of the syringe is then forced down. The entire procedure can be carried out with remarkable speed and safety and with a minimum of discomfort to the animal.

The stomach tube is best made from the head part of a French ureteral silk woven or x-ray catheter which is cut off so as to contain the rounded tip

and to be 9 cm. in length. We employed an Eynard x-ray catheter No. 9. A needle head (gauge 17) is attached to the tail end of the tube enabling connection to a syringe. A soft rubber catheter (No. 8 or 9 French) with rounded tip can be used as well if cut to the required length and fitted with a metal syringe adapter as shown in Fig. 1. The inexperienced should employ a soft rubber tube in preference to the stiff silk rubber or metal tube.

If assistance is available, maximum speed and efficiency can best be obtained by the following distribution of the required steps:

1. The operator immobilizes the animal by wrapping and inserts the *unattached* stomach tube into its mouth.

2. The assistant attaches the syringe and injects its contents into the animal's stomach (Fig. 2). He then withdraws the syringe and with it the stomach tube, detaches the latter, and fills the syringe for the feeding of the next animal. The stomach tube is best dropped into a container with water between feedings.

3. The operator, who in the meantime has wrapped and "gagged" another animal, recovers the moistened stomach tube and proceeds with the next intubation.

With a trained team of two, the entire feeding procedure requires less than half a minute per animal. Without assistance the time needed is somewhat longer.

SUMMARY

A simple "hairpin" gag is described which permits rapid and safe stomach-tube feedings of full-grown rats by a single person. With assistance and some experience, sixty rats can be intubated in thirty minutes.

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BOOK REVIEWS

Clinical Biochemistry. By *Abraham Cantarow*, M.D., Professor of Physiological Chemistry, Jefferson Medical College; formerly Associate Professor of Medicine, Jefferson Medical College, and Assistant Physician, Jefferson Hospital; and *Max Trumper*, Ph.D., Lieutenant Commander, H(S), U. S. N. R., Naval Medical Research Institute, National Naval Medical Center, Bethesda, Md.; formerly in charge of the Laboratories of Biochemistry of the Jefferson Medical College and Hospital. Third edition, revised. W. B. Saunders Company, Philadelphia, Pa., 1945. Price $6.50. Cloth with 647 pages and 29 illustrations.

The rapidity with which new developments in the field of biochemistry have accumulated in recent years has made it essential that new editions be written in which the many fundamental and clinical investigations may be correlated with clinical medicine. The present volume is an aim in this direction and it fills its role admirably.

While this book is in no sense a laboratory manual, it does bring together many facts of physiology and biochemistry related to clinical medicine and should be a valuable tool especially for the teacher and the clinician. To the laboratory worker in specialized fields, it presents a summarized view of much unfamiliar territory.

Newer methods of study in the fields of vitamins and a new chapter on Hormone Assay and Endocrine Function have been added since the second edition appeared five years ago. While exact technics of laboratory procedures are not included, the bibliography following each chapter provides references to the original literature where exact outlines of methods used in recent investigations may be obtained.

A table of normal clinical values is included and in the text one may find discussions relating to abnormal laboratory findings. These should be of immense value to the clinician.

On the whole, the book presents the conclusions of various investigators impartially and without personal interpretation, but in some instances the authors give their own opinion based on personal experience.

Any adverse criticism that the reviewer might make would be minor in nature. For example, the interchangeable use of the terms "glucose" and "dextrose" could well be avoided. It would seem that the effect of thyroxin on hepatic glycogen deserves more than mention since the effect of epinephrine is discussed rather fully. In discussing plasma proteins it would seem pertinent to call attention to other methods for determining specific gravity in addition to the falling drop method. Such methods as the copper sulfate and gradient tube methods for determining specific gravity might well be included since they are so valuable in the clinical laboratory.

The reviewer was particularly impressed with the review on lipid metabolism and the chapter on hepatic function. However, other subjects are also discussed with a thoroughness commensurate with the limitations of one volume.

The book is remarkably free of typographical errors and its printing and binding are admirable. As stated previously, the reviewer can recommend this work to the teacher, clinician, and laboratory worker alike and commends the authors on its excellence. A. E. O.

Frontiers in Chemistry (Vol. 4). Edited by *R. E. Burk*, Plastics Department, E. I. du Pont de Nemours & Co., Wilmington, Delaware, and *Oliver Grummitt*, Morley Chemical Laboratory, Western Reserve University, Cleveland, Ohio. Interscience Publishers, Inc., New York, N. Y. Price $3.50. Cloth with 151 pages.

Technique of Organic Chemistry. Edited by *Arnold Weissberger*. Interscience Publishers, Inc., New York, N. Y. Price $8.50. Cloth with 736 pages.

Textbook of Bacteriology. By *Edwin O. Jordan*, Ph.D., Late Andrew MacLeish Distinguished Service Professor of Bacteriology, University of Chicago; and *William Burrows*, Ph.D., Associate Professor of Bacteriology, University of Chicago. Fourteenth edition, revised. W. B. Saunders Co., Philadelphia. Price $7.00. Cloth with 909 pages and 242 illustrations.

Clinical Atlas of Blood Diseases. By *A. Piney,* M.D., M.R.C.P., Physician, St. Mary's Hospital for Women and Children; and *Stanley Wyard,* M.D., F.R.C.P., Physician, The Royal Cancer Hospital, London, and Princess Beatrice Hospital, London, Sixth Edition. The Blakiston Company, Philadelphia, Pa. Price $5.00. Cloth with 138 pages and 48 illustrations.

Lead Poisoning. By *Abraham Cantarow,* M.D., Associate Professor of Medicine, Jefferson Medical College; Assistant Physician, Jefferson Hospital; Biochemist, Jefferson Hospital, Philadelphia; and *Max Trumper,* Ph.D., Lieutenant Commander H-V (S) USNR, Naval Medical Research Institute, Bethseda, Md., Formerly Lecturer in Toxicology, Jefferson Medical College; Consultant in Industrial Toxicology, Cynwyd, Pa. Williams & Wilkins, Baltimore, Md. Price $3.00. Cloth with 264 pages.

Essentials of Histology. By *Margaret M. Hoskins,* Ph.D., and *Gerrit Bevelander,* Ph.D., Departments of Anatomy, The Graduate School of Arts and Science and College of Dentistry, New York University. The C. V. Mosby Company, St. Louis, Mo. Price $3.50. Cloth with 240 pages, 135 text illustrations, and 2 color charts.

Announcement

Due to conditions beyond the control of the editors and publishers, several issues of the JOURNAL are printed on an inferior grade of paper. Just as soon as the standard good grade of paper is available, its use will be resumed.